The role of thymic humoral factors in the maturation of rat lymphoid cells, assessed by response to PHA.

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ABSTRACT

The object of this thesis was to study the role of thymic humoral factors in the maturation of rat lymphoid cells.

The response, in vitro, of lymphoid cells to PHA, measured by the incorporation of tritiated thymidine, was used as a measure of thymic function.

Neonatal thymectomy led to a persistent lymphopenia and a drastically reduced response of rat whole blood and spleen cells to PHA. Partial restoration of the lymphocyte count and PHA response occurred in thymectomized rats following the transplantation of two syngeneic thymuses. Analysis of the PHA response and lymphocyte count in individual whole blood samples suggested that the response to PHA was a good measure of the peripheral blood T cell population.

Addition of serum to the medium was not necessary in order to obtain a satisfactory response to PHA provided that erythrocytes were present. No difference was found between the ability of pooled intact rat and pooled thymectomized rat serum to depress, or increase, tritiated thymidine incorporation in cultures of rat blood or spleen cells responding to PHA.

Incubation of unfractionated lymphoid cells with crude cell-free saline extracts of rat thymus was not found to increase the responsiveness of the cells to PHA. When the extract was present throughout the culture decreased isotope incorporation occurred. When the extract was removed from the cells after a preculture and before the addition of PHA the isotope incorporated in response to PHA was either slightly increased or decreased.

Incubation of unfractionated spleen, bone marrow and thymus cells with cell-free medium obtained from three-day and six-day thymus organ cultures did not lead to increased responsiveness to PHA. Also, 40 injections of thymus organ culture medium given to each of 12 neonatally thymectomized rats over an eight week period did not increase the whole blood response to PHA.

C O N T E N T S

C H A P T E R O N E

GENERAL INTRODUCTION. THE IMMUNOLOGICAL ROLE OF THE THYMUS.

INTRODUCTION

This thesis is concerned with an investigation into the immunological role of thymic humoral products. A review of past and recent studies into the function of the thymus is presented in chapter one where particular reference is placed upon possible endocrine functions.

Classically, suspected endocrine function has been investigated as follows. The organ is extirpated and the effects of extirpation are noted. Restoration is then attempted with organ extracts and/or transplants and finally isolation and characterisation of the active prinicple(s) is pursued. In the case of the thymus, recognisable deficits following thymectomy have only been described during the last 15 years. The immunological function of the thymus has been convincingly demonstrated by work during this period following the initial demonstration by Miller (Miller, J.F.A.P. 1961) and Good and his associates (Good, R.A. et al 1962) that neonatally thymectomized mice became lymphopenic and failed to reject allogeneic skin grafts.

HISTORICAL STUDIES

The first reference to the thymus is credited to Rufus the Ephesian in the first century B.C., whilst describing the anatomical discoveries made at the School of Alexandria under the Ptolemies (Keynes, G. 1954). A function for the thymus was first suggested by Galen in the second century A.D. who, because of the position of the gland in the anterior mediastinum, suggested that it was put there to protect the vena cava from coming into contact

with the hard surface of the sternum. Galen also regarded the thymus, because of its close proximity to the heart, as the centre of courage and affection. The word "thymus" is derived from the Greek "thymos" (Websters New International dictionary (1961)), however, it is not known whether the term was used because of the gland's supposed resemblance to the leaves of the thyme plant or because of its possible relationship to the spirit or courage, also designated meanings of the word. Thirteen centuries later, Vesalius drew and described the thymus which he thought acted as a cushion to protect the intra-thoracic organs (Crotti, A. 1938). In the middle of the seventeenth century, Glisson, impressed by the relative size of the thymus in foetal and neonatal life, suggested that the thymus might be connected with the growth of the foetus (Glisson, F. 1650).

The first important monograph on the thymus was published by Sir Astley Cooper in 1832 (Cooper, A. 1832). He investigated in detail the thymus glands of the calf and of the human foetus. In both of these he was impressed by the amount of a milky fluid that the gland seemed to contain and he suggested that the function of the thymus was connected with foetal growth since after birth the gland gradually decreases in size. Such a function was also suggested by Simon (Simon, J. 1845) who thought that the thymus produced a fluid secretion in early life that was connected with the nourishment of the animal.

I. Thymus extirpation studies.

A long series of thymus extirpation experiments beginning with those of Restelli in 1845 (Restelli, D.A. 1845) were undertaken with the expectation of producing some abnormality in the growth

and development of the animal. These experiments have been reviewed (Park, E.A. and McClure, R.D. 1919) (Hammar, J.A. 1921) (Crotti, A. 1938). Many of the conclusions drawn from the extirpation experiments, however, were invalid for the following reasons. Thymectomy was often incomplete. This was due to ignorance of the anatomical position of the thymus and a failure to check for thymic remnants. Conclusions were often drawn from the study of too few animals. Many of the deleterious consequences of thymectomy were undoubtedly non-specific effects of the operation, such as infection. Sham-thymectomies were not always performed to distinguish between these effects. Poor care of laboratory animals also contributed in several cases to the reported effects of thymectomy. Also, important details such as the age when thymectomy was performed was often not mentioned by the author.

Restelli operated on 98 animals including sheep, dogs and calves, only six animals survived and these died within 23 days from infections (Restelli, D.A. 1845). This work is therefore of historical interest only.

Friedleben removed the thymus from three goats and fifteen dogs (Friedleben, A. 1858). The goats were only partially thymectomized but seven dogs were thought to have been completely thymectomized. Three of the dogs died within two weeks, two lived for 17 and 47 days when they were killed and autopsied with no unusual findings. One dog killed after 22 days showed abnormalities at the ends of the long bones. His "prize" dog remained well until four months after the operation, whereupon it became emaciated, developed chronic diarrhoea with attacks of colic, and died. Friedleben suggested that the dog died as a result of neglect after

having passed out of his care. Further experiments in which thymectomy was combined with splenectomy all proved fatal and this suggested to Friedleben that the thymus and spleen were together essential organs. He concluded that the thymus was not essential to life but regarded it as an important organ whose functions were especially concerned with blood formation, nutrition and growth.

The next publication was that of Langerhans and Saveliew in 1893 (Langerhans, R. and Saveliew, N. 1893). They operated on 29 rabbits aged 3 - 5 weeks and two dogs. Some of their thymectomized rabbits survived at least two months and one completely thymectomized dog was alive and well ten weeks after the operation.

In 1894 Tarulli and Lo Monaco thymectomized two puppies and kept littermate controls (Tarulli, L. and Lo Monaco, D. 1894). Soon after the operation the haemoglobin content and the number of red cells in the blood decreased and a slight leukocytosis was noticed. The blood picture returned to normal after four months. The thymectomized dogs, despite having voracious appetites, put on weight less rapidly and appeared less vigorous than control (unoperated) dogs. Also, the hair of the former did not grow properly, became course, lost its lustre and could be pulled out easily. When Tarulli and Lo Monaco operated on 2 - 8 day old chickens (Tarulli, L. and Lo Monaco, D. 1897) most of them died within a week showing weakness of the legs, anaemia and an increase in white blood cells. Chickens operated on when 25 days old showed none of these symptoms. The authors concluded that deprivation of thymic function lowered the "resistance" of the animal for the first few days after the operation because several thymectomized

animals died without showing adequate cause at post mortem.

Abelous and Billard published the results of their experiments on frogs in 1896 (Abelous, J.E. and Billard, 1896). They found that no frog in which both thymus lobes were removed survived longer than 14 days. Symptoms usually appeared 24 - 48 hours after the operation and included lassitude, muscular weakness (leading to paralysis), loss of normal skin colour, ulcers, anaemia, leukocytosis and oedema. If, however, only one lobe was removed the animal did not die, unless it was in a debilitated state, and none of the severe symptoms developed. A subcutaneous thymus transplant and a calf thymic extract restored the skin colour but did not prevent death. They concluded that, in the frog, the thymus was essential to life and suggested that it produces an antitoxin which neutralises a toxin produced in the course of normal metabolism.

Though Camia (Camia, M. 1900) confirmed Abelous and Billard's results, others failed to do so. Ver Eecke observed that thymectomized frogs developed ulcers when kept in small stagnant tanks, whereas, when kept in clean water they did not (Ver Eecke, A. 1899 a b c). He therefore suspected that Abelous and Billard's results might have been due to death by infection from unchanged water in the tanks. Vincent found that extirpation of the thymus in frogs did not necessarily end fatally (Vincent, S. 1903 b). Some of his operated frogs survived for 36 days at which time they either died from causes not related to the operation or were killed. Pari isolated staphylococci and streptococci from the blood and organs of thymectomized frogs kept in stagnant water. He found that about one-third of completely thymectomized frogs succumbed to infection whereas only three out of sixty "incised" (sham-operated) frogs and three normal

frogs succumbed (Pari, G.A. 1905 a b) (Pari, G.A. 1906). Pari thought that the symptoms, described in thymectomized frogs by Abelous and Billard, were caused by infection, and were not the result of thymus deprivation per se. He, nevertheless, thought that the loss of the thymus was not without its effect and concluded that the thymus had antibacterial properties.

Hammar performed bilateral, unilateral or sham-thymectomy on 100 frogs. He found that the removal of either one or both thymic lobes produced no effect, whether the operation was performed in the spring or autumn, or whether the animals were fed or not (Hammar, J.A. 1905) (Hammar, J.A. 1910). Hammar found no signs of hypertrophy in the thymic remnants of his incompletely thymectomized frogs and in some cases found an involution of the thymic remnant. His work contained thorough histological checks in order to confirm the completeness of thymectomy and demonstrates that, in the frog, the thymus was not essential to life.

Adler attempted to remove the thymus from 950 tadpoles, however only 32 survivors remained at one week and 20 at 3 months (Adler, L. 1914). When the frogs were killed and examined the thymus was found to have been completely removed in only three frogs. None of the operated animals showed abnormalities in growth or development although the testes of the thymectomized frogs were larger than those of control frogs and the thyroid was hyperplastic. Adler concluded that, in the frog, a specific relationship exists between the thymus and both the thyroid and the testes. Allen in 1920 reported a series of thymectomies in Rana pipiens tadpoles (Allen, B.M. 1920). He agreed with Adler that the thymus was not essential for normal growth and development. However, although the completeness of thymectomy was confirmed at autopsy, no modifications

of the gonads, thyroid or other organs was found.

The last of a long series of reports on frogs was provided by Hoskins (Hoskins, M.N. 1921). This paper was composed by M.N. Hoskins from results of experiments carried out by her late father E.R. Hoskins. He had found that complete thymectomy in Rana sylvatica tadpoles had no effect on the size or histology of the spleen or on the growth and development of the larvae. Also, grafting thymus tissue into a tadpole did not effect the size or structure of the normal thymus, spleen or other organs.

Cozzolino thymectomized 24 rabbits, 14 of which survived (Cozzolino, O. 1903 a) (Cozzolino, O. 1904). No abnormalities were detected in these rabbits over a maximum observation period of 67 days. Cozzolino injected thymectomized and control rabbits with diphtheria toxin to gauge their resistance to the toxin, however he could make no distinction between the two groups. In another paper (Cozzolino, O. 1903 b), Cozzolino reported lesions in the foreleg of thymectomized rabbits and suggested a relationship between the thymus and the growth of bone. This latter study was not controlled by the presence of unoperated rabbits, however.

Paton and Goodall reported that thymectomy in the guinea-pig, even on the day after birth, had no influence on the growth of the animal. (Paton, D.N. and Goodall, A. 1904). A decrease in blood leukocytes was observed during the experimental observation period of two months. They noticed that if a thymectomized guinea-pig became pregnant a transient leucocytosis occurred. Resistance to the toxins from staphylococci and streptococci was diminished by thymectomy but no change was found in the resistance to diphtheria toxin.

Fischl in 1905 published an extensive report on thymectomy

carried out in goats, dogs and rabbits (Fischl, R. 1905). His negative findings, which included a study on the healing of fractures, led him to conclude that the thymus functions only during foetal life. This work was severely critisized by Park and McClure for its unsystematic presentation and lack of essential details (Park, E.A. and McClure, R.D. 1919).

Basch published several articles on experimental thymectomy between 1902 and 1908. These experiments have been reviewed in detail (Park, E.A. and McClure, R.D. 1919). Basch's own review of his thymus extirpation work was published in 1906 (Basch, K. 1906) and his report on the relationship between the thymus and the nervous system appeared in 1908 (Basch, K. 1908). After preliminary trials with rabbits, frogs, pigeons, guinea-pigs and cats he chose the dog as that most suitable for his purposes. He thymectomized puppies when three to four weeks old. After a further three to four weeks the operated animals appeared weaker, listless and bending of the bones was easily accomplished. The body weight of the thymectomized fell behind that of the controls and also the operated animals appeared dull and stupid. Basch described the bone changes as similar to that seen with rickets, and noted that after six months the animals showed normal skeletons. From his work on the nervous system Basch proposed that the thymus, along with the parathyroids, was intimately concerned with the etiology of tetany.

That the thymus had no essential function was the finding of Maclennan who actually reported beneficial effects when rabbits were thymectomized at six weeks of age (Maclennan, A. 1908). If the animal survived the operation it would recover within a few days to be indistinguishable from the rest of the brood. He reported that thymectomized animals grew faster and were more resistant to

disease. The thyroid in the thymectomized animals was smaller and lighter in colour, more cellular and had smaller glandular spaces. The author also advocated the use of thymectomy in humans as a therapeutic operation in cases of "thymic stridor".

Between 1906 and 1910 Soli carried out thymus extirpations on cocks, rabbits, guinea-pigs and pigs (Park, E.A. and McClure, R.D. 1919). Soli concluded that thymectomy had no effect on the skeleton if the development of the latter had progressed to a certain point. However, if thymectomy was done on young animals and they were killed shortly afterwards skeletal alterations could be seen. His work with hens was particularly interesting. The thymectomized hens passed through a period during which the eggs they laid had very thin shells or were without shells. This could be remedied by feeding calcium and magnesium carbonate.

Sommer and Florcken in a series of experiments using puppies and kittens found that two months after the operation thymectomized animals showed "awkward" behaviour, clumsy gait, roughness and dryness of the hair (Sommer, A. and Florcken, H. 1908). X-rays showed the skeleton to be normal, however. The animals were killed at six months of age and thymectomy was confirmed. At this time the long bones of the thymectomized animals were shorter and thicker, could be bent relatively easily and were deficient in calcium. The authors concluded that the thymus stimulates the growth of bone in post-foetal life.

Klose published an important work on the function of the thymus in conjunction with Vogt in 1910 (Klose, H. and Vogt, H. 1910). Klose was an extremely prolific experimenter and writer and was considered to have thymectomized twice as many animals as any previous investigator. Klose and Vogt used ten litters of

puppies, a total of 64 and kept one puppy from each litter as a control. If the animals were thymectomized 10 - 14 days after birth the following signs appeared 14 days to 3 months after the operation. The thymectomized animals gained weight at the same rate as the controls yet often ate twice as much. The thymectomized animals tired easily, their muscles tended to be replaced by fat and their long bones were more pliable. The intelligence of thymectomized animals was considered to be lower than that of control animals. For example, operated dogs often ate stones, had a dreamy gaze and showed a reduced sense of pain. They were also prone to muscle tremors, infections, distemper, pneumonia, diarrhoea and had dry hair which easily fell out. The thymectomized dogs passed into a coma ("coma thymicum") and died between $3\frac{1}{2}$ - $17\frac{1}{2}$ months after the operation. If thymectomy was performed at 3 - 4 weeks then the latent period before the onset of the signs was prolonged. Death was still inevitable but delayed up to two years. If the animals were operated on after four weeks then either no signs or transient signs occurred. Klose and Vogt thought that the thymus was involved with the nervous system and referred to the condition in thymectomized dogs as "thymicidiody". Thymectomized dogs had shorter long bones with reduced ossification. Chemical analysis showed the calcium content of thymectomized dog bones as being half the value found in control dogs. They also noticed hypertrophy of the spleen, thyroid, pancreas and genital organs following thymectomy, however microscopic analysis showed unaltered structure. They thought that the function of the thymus in foetal and early post-natal life was as the chief organ of "nuclein synthesis". When the thymus was removed phosphoric acid or perhaps nucleic acid was no longer neutralised and accumulated

giving rise to phosphoric acid acidosis. This acidosis was the cause of alteration in the bones (decalcification) and the oedematous condition of the brain.

Matti performed thymectomies on puppies aged between 18 days and 12 weeks and concluded that the thymus was an organ of internal secretion and was essential to life (Matti, H. 1911). Four weeks or longer after the operation the thymectomized animals became less active and tired more easily than controls. It became apparent that the legs of the thymectomized dogs were shorter and weaker and could easily be bent. Muscular weakness also developed and the weight of the thymectomized animals began to fall. At this stage some of the thymectomized animals were too weak to rise from the ground. Their hair became dry and coarse and death soon followed. No signs of diminished intelligence, abnormal appetite or an "adipose stage", as described by Klose and Vogt, were observed. Unlike Basch, Matti considered the bone changes permanent and described the condition as being identical or very similar to rickets. Matti's control animals remained healthy as did some of his thymectomized animals, for the latter he postulated the presence of functioning occult thymic remnants.

Pappenheimer in a study of the effect of thymus extirpation in rats weighing 10 - 25 g. (less than 21 days old) found no effect on growth and development (Pappenheimer, A.M. 1914 a). He performed sham-operations in some litters and checked for the presence of thymic remnants by tissue sectioning and microscopic examination. Pappenheimer found no change in the skeletal system, teeth, spleen, testes, adrenals or thyroid by histological examination. Pappenheimer also observed a decrease in blood lymphocyte count

which he followed for five weeks. He did not follow up this observation however. In another paper (Pappenheimer, A.M. 1914 b), Pappenheimer described an "epidemic" of rickets in his rat colony. The bones and teeth of both thymectomized and control animals were effected and he concluded that the loss of thymic function was not a valid explanation for the development of ricket-like bone lesions.

Renton and Robertson similarly failed to demonstrate any link between thymectomy and rickets (Renton, J.M. and Robertson, M.E. 1916). They operated on 17 dogs, 8 of which survived during the observation period and, in which, no thymic remnants were found. From 12 - 14 weeks an outbreak of "spontaneous" rickets appeared in the colony and both thymectomized and control dogs developed the disease in an identical manner. The spontaneous rickets gave rise to signs similar to those described by Basch, Klose and Vogt, and Matti. Renton and Robertson therefore suggested that thymectomy could not be responsible for the onset of rickets or similar bone changes.

Tangu extirpated the thymus gland from 40 dogs, the majority of which died soon after the operation from infections or shock (Tangu, Y. 1919). He found no changes in the growth and development, nervous system, skeletal system and in other secretory organs in any of his thymectomized animals.

In 1919 Park and McClure produced their detailed review of the literature on thymus extirpation (Park, E.A. and McClure, R.D. 1919). Their own research showed that the thymus was not essential to life in the dog. Thymectomy did not produce alteration of hair, teeth, body contour, muscle development, activity or intelligence in the experimental animal. They were

thus not able to confirm the link between thymectomy and defects in the skeletal system. The excellent work of Park and McClure turned the tide of opinion against the belief in the ricket-producing effect of thymectomy.

Renton extirpated the thymus from guinea-pigs weighing 100 g. and from rabbits at 15 - 20 days of age and found no effects from thymus deprivation (Renton, J.M. 1916). Similarly, Park found that guinea-pigs, thymectomized between the first and fifth days of life, showed no effects during an observation period of up to one year (Park, E.A. 1917). Park however, found that his thymectomies were not complete as accessory thymus lobes were found within the parathyroids.

Ackert and Morris studied the effect of thymectomy in growing chicks (Ackert, J.E. and Morris, M.H. 1929). They found no difference between thymectomized and control birds including no difference between the weight, strength and calcium carbonate content of the eggshells. Morgan and Grierson produced a similar report on the effect of thymectomy in young fowls (Morgan, A. and Grierson, M.C. 1930). They found that neither partial nor complete thymectomy appeared to effect normal living or growth, and there was no defect in calcium metabolism as judged by the production of normal eggs. Indeed, chickens made thymus-free continued a high production of perfect eggs during one year's observation after thymectomy.

Rowntree and associates while investigating the effects of a thymus extract, found that thymectomy in rats caused retardation in growth and body length curves (Rowntree, L.G. et al 1936). This effect was seen to increase through succeeding generations of thymectomized rats. Segaloff and Nelson investigated the claims of Rowntree and reported that thymectomy on the 21st day of life

in six successive generations did not alter the rate of growth in male or female rats (Segaloff, A. and Nelson, W.O. 1940). No difference was found in the development of their thymectomized and control rats.

Reinhard studied the results when Long-Evans rats were thymectomized on the day after birth and autopsied at 63 days together with sham-thymectomized littermate controls (Reinhard, W.O. 1945). He found no difference in body weights between the two groups and the only gross changes found were a decrease in the lymph node and spleen weights of the thymectomized rats.

In reviewing the literature on experimental thymectomy up until the middle of this century, a wide variety of effects have been described. This was due, in considerable part, to the reasons listed at the beginning of this section. In summary, removal of the thymus from the frog, rat, dog, rabbit, guinea-pig and chicken can be accomplished without the death of the animal. The confusion regarding the possible function of the thymus led Robert Leaton to state in 1946 that "No structure of the body is less understood, nor, with few exceptions, has had more functions attributed to it, than the thymus". (Leaton, R.E. 1946). It is clear that many investigators have placed over-valuation on positive experimental results at the expense of negative results. Those workers who did this were perhaps convinced that positive results would result from thymus extirpation. In anticipating the role of the thymus in host immunity it is difficult not to be impressed by the number of animals dying from infections and wasting diseases. This was due in considerable part no doubt, to non-aseptic operating conditions and poor housing, but also perhaps

to immunological deficiency.

Finally, in considering the results on skeletal alterations and the calcium content of bones it might have been possible that too clumsy a thymectomy damaged or removed the parathyroids which are responsible for the synthesis of parathyroid hormone and calcitonin. Furthermore, Galante in 1968 isolated calcitonin, from the human thymus (Galante, L. et al. 1968). However, whether the thymus has a function in calcium metabolism or whether the presence of calcitonin reflects the close embryological origin of certain cell types in the thymus and thyroid is not known. Thymic extracts with hypocalcaemic action have also been described (Nitschke, A. 1928) (Scholtz, H.G. 1932) (Mizutani, A. et al 1970).

II. Relationship between the thymus and the gonads.

The idea of a physiological relationship between the thymus and the sex organs arose from the observation that thymus involution seemed to occur at or around the time of puberty. The following lines of research have been pursued:

(1) The effect of various phases of normal sex life, such as puberty, mating, pregnancy and lactation on the weight of the thymus.

(2) The effect of removal of the gonads on the thymus.

(3) The effect of thymectomy on sexual anatomy and physiology.

(4) The effect on the thymus of administration of "gonadal substances" by feeding and injection.

(5) The effect on the gonads of administration of thymus substances, etc.

The literature on this topic is often contradictory as it

failed to take into account the normal variation found in animals and that malnutrition and infection reduced the weight of the thymus and gonads. A comprehensive review on the relationship between the thymus and reproduction was published in 1932 by Dorothy Andersen (Andersen, D.H. 1932). She concluded that thymectomy had no effect on the weight, morphology and physiology of the gonads, except through trauma or infection from the operation. The gonads, however, may exert some control over the thymus as pre-pubertal castration delayed thymus involution.

A more comprehensive discussion of this topic is outside the scope of this review.

III. Thymus feeding experiments.

These experiments were carried out to try to demonstrate that the thymus contained an active principle which, when given in excess, would produce a state of "hyperthymusization".

In 1914 Gudernatsch reported that tadpoles which had been fed exclusively on calf thymus showed an accelerated growth rate, and an increased body size (Gudernatsch, J.F. 1914). At the same time metamorphosis was either retarded or completely suppressed. Tadpoles fed thyroid gland metamorphosed without undergoing a growth phase. That thymus feeding retarded metamorphosis was confirmed by Romeis, (Romeis, B. 1915). Later, Romeis (Romeis, B. 1925) and Abderhalden (Abderhalden, E. 1926) suggested that the retarding effect of thymus substance was not due to a specific hormone but to the absence of some factor necessary for metamorphosis. Uhlenhuth reported that Salamander larvae (Amblystoma opacum), when fed on thymus soon after hatching, developed tetanic convulsions at 35 - 40 days (Uhlenhuth, E. 1918 a b c). He

thought that the thymus produced an internal secretion which was capable of producing tetany in the larvae and which could be antagonized by the parathyroids. It seems reasonable to suspect that the tetanic convulsions were caused by a diet that was not sufficient.

The first mammalian experiments to appear were those of Hewer (Hewer, E.E. 1914). She fed rats with thymus tabloids (Burroughs and Wellcome) or fresh lamb thymus, plus a little milk. Hewer reported that thymus feeding delayed the development of the testis in young male rats and in older rats caused degeneration of the testis. The work of Hoskins, also on rats, showed no effect on the gonads after thymus feeding (Hoskins, E.R. 1916). Romeis reported a delay of spermatogenesis and a decrease of testis size in rats fed on an exclusive thymus diet. (Romeis, B. 1926). If the thymus diet was supplemented with vegetable food, however, the rats and their sex glands were normal. These experiments therefore indicated that the effects, observed in animals fed an exclusive, or predominantly, thymus diet, were caused by dietary insufficency.

IV. Thymic extracts.

Most of the important studies of thymic extracts reported prior to 1949 are detailed in table 1.1. In 1949, Roberts and White showed that a calf thymic extract, when injected into rats, produced a marked lymphocytosis and stimulation of the lymphoid tissue (Roberts, S. and White, A. 1949). Some earlier workers had noticed the effects of thymic extracts on leukocytes but the major effect on the lymphoid system, as reported by Roberts and White can be considered a turning point towards the modern interpretation of the function of the thymus.

One of the first descriptions of the effect of a thymic extract was that by Brieger and co-workers (Brieger,et al 1892). They found that certain bacteria showed a reduced virulence when cultivated in a thymic extract or when a thymic extract was added to the culture medium. They also injected animals simultaneously with thymic extract and suspensions of bacteria such as tetanus and cholera, and found that the thymic extract reduced the virulence of these organisms. Barbara reported that both thymic and thyroid extracts stimulated the phagocytic properties of leukocytes (Barbara, M. 1918). Antibacterial effects of a thymic extract have been described more recently (Dubos, R.J. and Hirsh, J.G. 1954) (Li, C.P. et al 1963). However other organ extracts, besides the thymus, show antibacterial activity and it is most likely that such effects as described by Brieger and Barbara were non-specific.

Many experimenters have described effects of thymic extracts which were undoubtedly not specific to the thymus gland. For example, the lowering of blood pressure that occurred following intravenous injection of thymic extract (Svehla, K. 1896) (Vincent, S. 1903 a) (Basch, K. 1913). In 1918 Olkon reported an experiment in which he had injected growing guinea-pigs with desiccated sheep thymus suspended in physiological saline (Olkon, D.M. 1918). These injections had produced muscle spasms, convulsions and in some animals death. Animals injected with thymic substance failed to gain weight or even lost weight. Desiccated muscle protein produced similar effects although not so severe as those due to the thymic substance. A similar experiment was performed by Downs and Eddy. They injected a desiccated thymic extract, resuspended in glycerine,

and saline (1:4), subcutaneously into young rabbits. Large doses limited the weight gain of rabbits and led to a large increase in the weight of thyroid and spleen and a decrease in the weight of the thymus (Downs, A.W. and Eddy, N.B. 1920) Intravenous injections of thymic extracts have also been reported to inhibit the occurrence of, or even to neutralize, muscle fatigue (Muller, H. 1917) (Del Campo, E. 1918) (Thurner, K 1924). The extract was thought to act on the nerve and not the muscle.

An effect of thymic extracts on calcium metabolism has been the finding of several workers (Bracci, C. 1905) (Nitschke, A. 1928) (Scholtz, H.G. 1932). Bracci found that rabbits showed increased calcium excretion after the thymus had been removed, but in those animals injected with a thymic extract a lowered calcium excretion was found. Work on a hypocalcaemic component extracted from bovine thymus is still in progress (Mizutani, A et al 1975) and this aspect of thymic function is still unclear.

A group of French workers (Camus, J. and Gournay, J.J. 1924) (Lereboullet, P. and Gournay, J.J. 1927) (Mandelstamm, M. 1927) reported a few cases in which delayed development of the testes in dogs and men had been cured by injections of thymic extract.

A combination of extracts of the thymus and pituitary, known as Thymophysin, has been used to strengthen uterine contractions during labour. This subject has been reviewed by Jarcho (Jarcho, J. 1930). In 1926, Temesvary described his experiments using isolated uterine segments (Temesvary, N. 1926). He found that thymic extract alone, in large doses, increased uterine contractions slightly and that this effect was much increased by the addition of a pituitary extract. Other workers found that the thymic constituent of Thymophysin could be replaced by other organ extracts without

reducing the effect of the combined extract (Jarcho, J. 1930). Present day knowledge attributes the effect of Thymophysin to the presence of oxytocin from the pituitary.

In 1930 Leon Asher reported that an extract of calf thymus which he called Thymocrescin, had a stimulating effect upon the growth and sex organs, of rats (Asher, L. 1930). This report was followed by those of various associates of Asher, (Nowinski, W. 1930) (Stotzer, P. 1931) (Bachmann, H. 1934) and Asher (Asher, L. 1936). Thymocrescins were prepared by treating calf thymus with acetone and ether, followed by extraction with water, fractional precipitation and further extractions with alcohol, water and ammonium sulphate. The active fractions were thought to be polypeptides which contained sulphur. Small amounts of Thymocrescin injected each day into young rats was found to accelerate the growth rate, stimulate the gonads and hasten puberty. Control extracts prepared from lymph nodes were inactive.

In 1934 Rowntree and his collaborators, Clark and Hanson, reported the first of a series of experiments on the biological effects of a calf thymus extract (Rowntree, L.G. et al 1934). The material used initially in these studies had been prepared by Dr. A.M. Hanson by extracting calf thymus for several hours in a 0.5% solution of hydrochloric acid at temperatures approaching 96°C. Hanson called this extract "Karkinolysin" and reported that four cases of inoperable carcinoma when treated daily by injections of extract showed some degeneration of the tumour (Hanson, A.M. 1930). In 1933, Rowntree began to treat rats with Hanson's extract. The extract had been prepared in 1930 and had been stored at room temperature during this interval. Unlike the experiments with Thymocrescin, treatment with Hanson's extract did not produce any

dramatic results in the first and second generations. In the third and later generations, however, the rate of growth and differentiation of the rats became accelerated up to a saturation point. Processes such as ear opening, teeth eruption, appearance of hair, eye opening, descent of testes and vaginal opening occurred earlier. The fertility of the offspring of thymus-treated parents was also considerably enhanced. Thymectomy over successive generations was reported to produce a retardation in the growth and development of rats (Rowntree, L.G. et al 1936). It was found that injection of thymic extract reversed the effects of thymectomy and also that thymus implants into thymectomized rats could similarly restore growth and development to normal (Rowntree, L.G. et al 1936). (Einhorn, N.H. 1938). The Hanson extract was found to have a high sulphur content and to be rich in iodine-reducing compounds. In later experiments Rowntree and co-workers found that fresh solutions of glutathione, ascorbic acid and cysteine could simulate some of the effects of thymic extract (Rowntree, L.G. et al 1938).

Rowntree's results, however, were not confirmed although many workers tried to repeat his experiments (Smith, G.V.S. and Jones, E.E. (1940) (Allardyce, J. et al 1940) (Segaloff, A. and Nelson, W.O. 1941) (Burrill, M.W. and Ivy, A.C. 1941). The most likely explanation for the positive results obtained by both Asher's and Rowntree's groups was that growth was stimulated by injections of pharmacological agents. Also, perhaps the results reflected a desire to demonstrate a role for the thymus.

The experiments of Bomskov have been critically reviewed (Andreasen, E. 1946). Bomskov extracted a fat-soluble product from the calf thymus with the following properties:

1. mobilises glycogen from the liver and heart.

2. stimulates growth in hypophysectomized rats.
3. produces lymphocytosis and leukocytosis.
4. causes regression of the gonads, especially in the male.
5. reverses the effects of thymectomy in three-day-old guinea-pigs (thymectomy led to arrest of growth, loss of muscle tone and muscle atrophy leading to death.)

This work was not confirmed by other workers except with respect to the lymphocyte stimulating effect (Rehn, E. 1940).

Table 1.1 Thymic extracts and their reported biological effects.

Year.	Authors.	Type of extract.	Biological effect.
1892	Brieger,[a] Kitasato and Wasserman	NS[b]	Anti-bacterial effect
1896	Abelous, J.E. and Billard.	Calf Thymus.	Restored skin colour and muscle tone in thymectomized frogs.
1896	Svehla, K.[c]	NS	Intravenous injection led to tachycardia and fall in blood pressure in dogs.
1903	Vincent, S.	Calf thymus, boiled in 9% saline, extracted with alcohol and ether.	Intravenous injection led to fall in blood pressure.
1905	Bracci, C.	Rabbit thymus, aqueous extract.	Stimulation of calcium metabolism and reduced calcium excretion in thymectomized rabbits.
1917	Muller, H.[a]	NS	Revitalised fatigued frog muscle.
1918	Del Campo, E.[a]	NS	Revitalised fatigued rabbit muscle.
1918	Olkon, D.M.	Desiccated sheep thymus in saline.	Loss of weight in growing guinea-pigs, muscular spasms and convulsions.

Year	Authors	Type of extract	Biological effect
1918	Olkon, D.M.	Desiccated sheep thymus in saline.	Loss of weight in growing guinea-pigs muscular spasms and convulsions
1918	Barbara, M.[a]	NS	Stimulation of phagocytosis.
1920	Downs, A.W. and Eddy, N.B.	Desiccated thymus resuspended in glycerine and saline	Large doses reduced weight gain in young rabbits.
1924	Thurner, K.	Thymus homogenate boiled in saline or 95% alcohol.	Revitalised fatigued guinea-pig muscle.
1924	Camus, J. and Gournay, J.J.[b]	NS	"Curing" delayed development of testes in man and dog.
1927	Lereboullet, P. and Gournay, J.J.[c]		
1927	Mandelstamm, M.[c]		
1926	Temesvary, N.	"Thymophysin" (thymus) and pituitary extract)	Increased force of uterine contractions during labour.
1929	Nitschke, A.	Acetic acid extract of calf thymus	Lowers serum calcium increases skeletal calcium, reduces serum phosphate.
1930	Asher, L.	Calf thymus, aqueous extract "Thymocrescin".	Stimulates growth in young rats.
1930	Hanson, A.M.	Calf thymus, dilute acid extract. "Karkinolysin".	Anti-tumour effect.
1934	Rowntree, L.G. Clark, J.H. and Hanson, A.M.	Hanson's extract and calf thymus extracted with sodium chloride at 68°C., precipitate discarded and pH adjusted to 3.5	Increased growth rate and fertility in rats.

Table 1.1 continued

Year	Authors	Type of extract	Biological effect
1940	Bomskov, C.	Calf thymus, lipid extract.	Mobilised glycogen, produced leukocytosis and lymphocytosis, caused regression of gonads.
1940	Rehn, E.	Calf thymus, lipid extract.	Caused lymphocytosis in man.
1947	Torda, C. and Wolff, H.G.	Cat thymus, extracted with acetone and ether.	Depressed acetylcholine synthesis.
1949	Constant, G.A. et al.	Suspension in saline.	Decreased muscle contraction in cat nerve-muscle preparation.

a. Quoted by Hammar, (Hammar, J.A. 1921).

b. NS Not specified.

c. Quoted by Olkon, (Olkon, D.M. 1918).

THE EFFECTS OF NEONATAL THYMECTOMY.

The first convincing demonstration that the thymus was an organ of vital importance was provided by Miller (Miller, JF.A.P. 1961) and Robert Good and associates (Good, R.A. et al 1962) who surgically removed the thymus from laboratory mice within 24 hours after birth. Thymectomy had previously been carried out in young and adult animals and the lymphoid system was mature by the time thymectomy was performed. Also, the period of observation was often not long enough to detect the gradual decline in immunological competence that occurs in adult animals following thymectomy. The consequences of neonatal thymectomy can be grouped under three broad headings:

(1) The failure of normal body growth and early mortality (the wasting syndrome).

(2) Lymphocyte depletion.

(3) Immunological deficits.

Each of these topics are related to one another but will be described separately for simplicity.

I. The wasting syndrome

The incidence and time course of the disease has been found to vary from laboratory to laboratory, between different species and between different strains of the same species. In general, mice appear to be more susceptible to wasting following thymectomy than most other laboratory animals. The disease may take the following typical course. Mice, thymectomized at birth, appear normal and gain weight at the same rate as their intact littermates. Between three and six weeks after birth, however, they start to lose weight, their coat condition becomes poor, the animals sometimes

became hunch-backed and walk with a high stepping gait, and often have diarrhoea (Miller, J.F.A.P. 1962 c) (Miller, J.F.A.P. 1962 b) (Parrott, D.M.V. 1962) (Parrott, D.M.V. and East, J. 1964 b) (Law, L.W. et al 1964 b). Once wasting starts it is usually a matter of, at most, a couple of weeks before the animal dies. The most obvious post-mortem features of the wasted mouse are the thinness of the skin and bone, and the lack of subcutaneous fat. Necrosis of the liver was found to occur in about half of the mice with granulamatous foci with aggregates of polymorphonuclear leukocytes, macrophages and giant cells (East, J. et al 1963) (Law, L.W. et al 1964 b). These lesions have been associated with the presence of a hepatotrophic virus (East, J. et al 1963), but there is no evidence that wasting or death results from this infection. The spleen and lymph nodes of neonatally thymectomized mice varied a great deal in size but were usually smaller and contained fewer germinal centres than lymphoid organs from unoperated mice, Attempts to arrest the progression of the disease by the administration of high protein or high vitamin diets or by doses of broad-spectrum antibiotics were not successful (Miller, J.F.A.P. 1964 b).

The earlier in life that thymectomy was performed the higher was the incidence of wasting in all the strains of mice studied (Miller, J.F.A.P. 1963 b) (Miller, J.F.A.P. 1964 b) (Law, L.W. 1966 a) Thymectomy at birth, or before birth by caesarean thymectomy, led to almost 100% wasting, while thymectomy performed one week after birth was not associated with significant mortality from wasting (Miller, J.F.A.P. 1964 b). Although the mean age at death did not vary widely, specific strain differences were evident. For example, most of the C57BL and A strain mice developed signs of

wasting by four weeks and were dead by two months, whereas (Ak x T6) F_1 hybrids began wasting between two and four months of age and about 75% of the mice had died four months after birth. (Miller, J.F.A.P. 1964 b).

Wasting has been reported in rats thymectomized at birth (Jankovic, B.D. et al 1962) (Azar, H.A. 1964) (Messini, M. et al 1964) (Aisenberg, A.C. and Wilkes, B. 1965) (Borum, K. 1974), however rats appear to be less susceptible than mice to develop the wasting syndrome. Neonatally thymectomized hamsters have also been found to undergo wasting (Sherman, J.D. et al 1963) (Adner, M.M. et al 1965) (Roosa, R.A. et al 1965) (Sherman, J.D. 1967 b). In one investigation the wasting syndrome was restricted to male hamsters (Sherman, J.D. et al 1963), however the other investigations showed no sex differences. The rabbit, an animal with a comparatively mature lymphoid system at birth, has not been found to waste after neonatal thymectomy (Archer, O. et al 1964). Wasting has been observed however, in thymectomized rabbits following total body irradiation (Kellum, M.J. and Eckert, E. 1965).

The cause (or causes) of the post-thymectomy wasting disease have not yet been clearly defined. Since the wasting syndrome closely resembled the graft-versus-host syndrome and is associated with lymphoid atrophy and immunological deficiencies, the wasting disease has been attributed to an autoimmune process (Miller, J.F.A.P. and Howard, J.G. 1964) (DeVries, M.J. et al 1964). The pathogenesis of the post-thymectomy wasting disease is debatable and experimental evidence has accumulated which supports the concept that the wasting syndrome is precipitated by infectious agents. Briefly, the experimental evidence supporting this concept is a follows:

(1) The incidence of wasting varies from one laboratory

to another. Wasting does not occur in all neonatally thymectomized mice (Hess, M.W. et al 1963). Jankovic reported that in the case of neonatally thymectomized rats, most litters did not succumb to the disease but in a few litters, all the rats wasted (Jankovic, B.D. et al 1962).

(2) Neonatally thymectomized mice and rats were found to be more susceptible than sham-operated controls to the following infectious agents: hepatotrophic viruses (East, J. et al 1963), herpes simplex virus (Mori, R. et al 1965), pyogenic bacteria (Azar, H.A. 1964), mycobacterium leprae (Rees, R.J.W. 1966) (Fieldsteel, A.H. and McIntosh, A.H. 1971), Candida albicans (Salvin, S.B. et al 1965) and to the endotoxins from E. Coli and S. typhosa (Salvin, S.B. et al 1965).

(3) Antibiotic treatment reduced the incidence of sepsis and wasting in neonatally thymectomized rats (Azar, H.A. 1964).

(4) Neonatally thymectomized pathogen-free mice did not develop signs of wasting (Hess, M.W. et al 1963) (McIntire, K.R. and Sell, S. 1964) (Hess, M.W. and Stoner, R.D. 1966).

(5) Neonatally thymectomized germ-free mice, maintained in a germ-free environment showed no signs of wasting (Wilson, R. et al 1964) (McIntire, K.R. and Sell, S. 1964) (Miller, J.F.A.P. et al 1967). When these mice were exposed to a normal environment, wasting developed only in thymectomized mice.

In spite of the above evidence that the wasting disease is the result of infectious factors no absolute proof has been obtained to confirm this. It has proved very difficult to isolate a pathogen from the tissues of animals suffering from the wasting syndrome. Bacterial cultures from wasted animals have shown the same microbial flora as those from non-thymectomized controls (Sherman, J.D. et al 1963) and attempts to isolate viral agents has not led to a satisfactory aetiological explanation.

II. Lymphocyte depletion.

Thymectomy carried out within a few days after birth results in reduced numbers of lymphocytes in the blood, spleen and lymphoid tissue in mice (Miller, J.F.A.P. 1961) (Miller, J.F.A.P. 1962 c) (Parrott, D.M.V. 1962) (Parrott, D.M.V. and East, J. 1964) (Kalpaktsoglou, P.K. et al 1969), rats (Waksman, B.H. et al 1962), hamsters (Roosa, R.A. et al 1963) (Adner, M.M. et al 1965), guinea-pigs (Ernstrom, U. 1965), rabbits (Sutherland, D.E.R. et al 1964), opossums (Miller, J.F.A.P. et al 1965 a), sheep (Morris, B. 1973) and chicken (Warner, N.L. and Szenberg, A. 1962) (Jankovic , B. and Isakovic, K. 1964). In normal mice the lymphocyte count and the lymphocyte to polymorphonuclear leukocyte cell ratio increases progressively from birth to adult levels with ten days (Miller, J.F.A.P. 1961). However, in neonatally thymectomized mice there was no increase in lymphocyte numbers. Total white blood cell counts in neonatally thymectomized mice were approximately one-half those of normal mice, and this diminution was due primarily to a decrease in the small lymphocyte population (Miller, J.F.A.P. 1961) (Miller, J.F.A.P. 1962 c) (Miller, J.F.A.P. 1964 b). After several

weeks the lymphoid organs of the neonatally thymectomized animals began to lose weight, the organs contained reduced numbers of lymphoid follicles and germinal centres. The most dramatic change was the severe depletion of small lymphocytes from the central areas of the spleen lymphoid follicles immediately surrounding the central arterioles and in the mid and deep cortical zones of the lymph nodes - the so called thymic-dependent areas (Parrott, D.M.V. 1966). Young neonatally thymectomized mice usually had lymphoid follicles and germinal centres but these became less active with age and were often not present in older thymectomized mice. There was no deficiency of plasma cells, which tended to accumulate in the thymic-dependent tissues in older thymectomized animals (Miller, J.F.A.P. and Osoba, D. 1967). In addition to the lymphocyte deficiency, neonatally thymectomized mice often showed hyperplasia of the reticuloendothelial elements, and increased numbers of reticulum cells, histiocytes and macrophages were present in the lymph nodes, spleen and liver (Miller, J.F.A.P. and Howard, J.G. 1964) (Schooley, J.C. et al 1965).

Results of thymectomy carried out on animals aged beyond two or three days, and especially in adult animals are not as dramatic as those in neonatal animals. These results are described in the section on adult thymectomy.

III. Immunological competence.

The development of immunocompetence is a process that is dependent upon both foetal and postnatal development of the primary and secondary lymphoid tissues. The secondary lymphoid tissue (spleen, lymph nodes etc.) of mice and rats is not fully developed until several days after birth and following thymectomy the thymic-

dependent tissues cease further development.

Studies of the effect of thymectomy and bursectomy in the chicken and thymectomy in the mouse have led to the identification of two functional groups of small lymphocyte (Cooper, M.D. et al 1965). Cells which develop under the influence of the thymus (T cells), participate in cell-mediated immunological reactions, and B cells, which develop in the bursal environment in birds and in an equivalent site in mammals function in humoral immune responses. The defects in immunological performance in neonatally thymectomized rodents have been evaluated by comparison with unoperated and sham-thymectomized rodents and these defects are discussed under the following subtitles:

(A) Effect on antibody production and immunoglobulin levels.

(B) Effect on cell-mediated immune responses.

(A) Effect on antibody production and immunoglobulin levels.

The finding that neonatal thymectomy impaired antibody responses to some antigens (Miller, J.F.A.P. 1962 b) was at first difficult to reconcile with the concept of a dual immune system. It then became apparent, however, that T cells were required in order for B cells to produce an optimum antibody response to certain types of antigen - thymus dependent antigens (Miller, J.F.A.P. and Mitchell, G.F. 1969).

Neonatally thymectomized mice were found to have depressed primary antibody responses to the following antigens: sheep erythrocytes (Humphrey, J.H. et al 1964) (Osoba, D. and Miller, J.F.A.P. 1964) (Fahey, J.L. et al 1965) (Schooley, J.C. et al 1965) (Miller, J.F.A.P. et al 1966), Salmonella typhi H.O. and Vi antigens (Miller, J.F.A.P. 1962 b) (Miller, J.F.A.P. et al 1962) (Humphrey, J.H. et al

1964), influenza A virus (Miller, J.F.A.P. et al 1962), T_2 coliphage (Good, R.A. et al 1962), ovalbumin (Arnason, B.G. et al 1964), bovine serum albumin (Arnason, B.G. et al 1964 a) (Basch, R.S. 1966), and diphtheria toxoid (Arnason, B.G. et al 1964 a). Normal or near normal antibody responses were found in response to the following antigens: tetanus toxoid (Hess, M.W. et al 1963), haemocyanin (Humphrey, J.H. et al 1964) (Fahey, J.L. et al 1965), pneumococcus type III capsular polysaccharide (Humphrey, J.H. et al 1964), ferritin (Fahey, J.L. et al 1965) and polyoma virus (Miller, J.F.A.P. et al 1964 b). Neonatally thymectomized rats were found to have reduced primary antibody responses to bovine serum albumin (Jankovic, B.D. et al 1969), flagellin from Salmonella adelaide (Steward, J.P. 1971) and sheep erythrocytes (Borum, K. 1972). It appears, however, that there are strain differences in the ability of neonatally thymectomized rats to respond to sheep erythrocytes as certain strains showed no reduction in antibody response following neonatal thymectomy (Pinnas, J.L. and Fitch, F.W. 1966) (Steward, J.P. 1971) (Borum, K. 1972). Rabbits, thymectomized between birth and five days, showed a reduced precipitin production to bovine serum albumin when the animals were tested at seven to eight weeks of age (Archer, O. and Pierce, J.C. 1961) (Archer, O.K. et al 1962). When thymectomy was delayed beyond five days after birth no depression in the response to BSA was observed. The response of neonatally thymectomized rabbits to bacteriophage T_2 was also reduced (Good, R.A. et al 1962). Neonatal thymectomy in chickens produced no consistent effect on antibody production (Warner, N.L. and Szenberg, A. 1962) (Graetzer, M.A. et al 1963) (Isakovic, K. and Jankovic, B.D. 1964). However, either surgical bursectomy at hatching or hormonal bursectomy in ovo severely impaired the ability

to produce an antibody response (Glick, B. et al 1956) (Mueller, A.P. et al 1960) (Mueller, A.P. et al 1962) (Papermaster, B.W. et al 1962) (Graetzer, M.A. et al 1963) (Isakovic, K. et al 1964) (Cooper, M.D. et al 1965).

The nature of the reduced antibody response to sheep erythrocytes in neonatally thymectomized mice has been investigated using the Jerne plaque assay (Jerne, N.K. and Nordin, A.A. 1963). This assay enables the number of cells responding to sheep erythrocytes in the spleens of the experimental animals to be counted since each responding cell gives rise to a single plaque. Independent studies (Takeya, K. et al 1964) (Takeya, K. and Nomoto, K. 1964) (Friedman, H. 1965) Miller, J.F.A.P. et al 1965 b) demonstrated that the spleens of erythrocyte-sensitized neonatally thymectomized mice contained very much fewer plaque forming cells than the spleens of sham-thymectomized, immunized mice, and that the antibody forming capacity of single spleen cells was not impaired in the absence of the thymus.

Conflicting reports are available concerning the ability of neonatally thymectomized mice to mount a secondary immune response. The primary response to sheep erythrocytes has been reported to be depressed, whilst the secondary response was nearly normal (Rogister, G. 1965) (Sinclair, N.R.S.C. 1967). Other reports (Hess, M.W. et al 1963) (Hess, M W. and Stoner, R.D. 1966) (Basch, R.S. 1966) indicated that the ability to produce an anamnestic response was more affected by neonatal thymectomy than was the primary response.

The capacity of neonatally thymectomized animals to synthesize immunoglobulins has been studied and reduced, normal and raised levels have been reported (Humphrey, J.H. et al 1964) (Arnason, B.G. et al 1964 b) (Fahey, J.L. et al 1965). Humphrey and co-workers found

that neonatally thymectomized mice took longer to recover from the physiological hypogammaglobulinaemia present three to four weeks after birth, but if the animals survived they developed near normal gammaglobulin levels after six weeks of age (Humphrey, J.H. et al 1964). In wasting neonatally thymectomized rats raised serum IgG levels were reported (Azar, H.A. 1964). However, the immunoglobulins of neonatally thymectomized animals appeared to differ only quantitatively and not qualitatively from those of unoperated controls.

The variation in the design of experiments investigating the influence of thymectomy on antibody production has been so great that it is very difficult to compare and evaluate the results. However, in summary, it appears that the antibody response of neonatally thymectomized animals is influenced by the animal species, age at thymectomy, nature of antigen and the immune response evaluation system. The primary and secondary antibody responses were either normal or depressed.

(B) Effect on cell-mediated immune responses.

In mice, thymectomized at birth there was a marked impairment in the animal's capacity to reject skin grafts regardless of the magnitude in histocompatibility difference between donor and recipient (Miller, J.F.A.P. 1961) (Good, R.A. et al 1962) (Miller, J.F.A.P. 1964 b) (Goedbloed, J.F. and Vos, O. 1965). Defective homograft rejection mechanisms were already evident at the age of three days in neonatally thymectomized mice whilst sham-operated littermates were able to reject skin homografts at that age (Miller, J.F.A.P. 1964 b). The longer the time interval between birth and thymectomy the smaller was the magnitude of immunological deficiency. Thymectomy between the third and seventh day of life resulted in impairment of homograft

immunity only when the histocompatibility difference between donor and recipient was slight (Miller, J.F.A.P. 1962c) (Good, R.A. et al 1962) (Dalmasso, A.P. et al 1963). A slight deficiency in allograft immunity was detected in mice thymectomized at 35 days of age (Dalmasso, A.P. et al 1962 b). Similarly, in rats thymectomized at birth an impaired ability to reject allogeneic skin grafts was found (Arnason, B.G. et al 1962 b) (Fischer, E.R. and Fischer, B. 1965). Also, neonatally thymectomized hamsters (Sherman, J.D. et al 1964) (Roosa, R.A. et al 1965) and chickens (Warner, N.L. and Szenberg, A 1962) (Aspinall, R.L. et al 1963) (Jankovic, B. and Isakovic, K 1964) showed prolonged acceptance of foreign skin grafts.

It has been emphasized that skin allograft rejection is usually complicated by the post-thymectomy wasting syndrome and that large numbers of mice can die from wasting during the experimental observation period (Hess, M.W. 1968). Skin graft experiments carried out using germ-free mice showed that skin graft rejection, although impaired in neonatally thymectomized germ-free mice, was not impaired to the extent seen in conventional neonatally thymectomized mice (Miller, J.F.A.P. et al 1967). Miller concluded that the difference in rejection times might be due to environmental factors operating in the conventional state, such as bacterial contamination, endotoxins and cross-reacting antigens which would tend to reduce even further the number of antigen-reactive cells available in an already limited pool of immunologically competent cells.

Neonatal thymectomy has been found to render animals more susceptible to the oncogenic effects of several tumour viruses (Miller, J.F.A.P. et al 1964 b)(Defendi, V and Roosa, R.A. 1964) (Defendi, V. and Roosa, R.A. 1965) (Allison, A.C. and Taylor, R.B. 1967) (Van Hoosier, G.L. et al 1968). Neonatal thymectomy also impairs the ability of an

animal to reject allogeneic and xenogeneic tumour transplants (Martinez, C. et al 1962) (Mcentegart, M.G. et al 1963) (Parrott, D.M.V. and East, J. 1965) (Osoba, D. and Auerspera 1966) (Kubista, T.P. et al 1967) (Trainin, N. and Small, M. 1970).

Cells obtained from the lymphoid organs of neonatally thymectomized mice (Miller, J.F.A.P. et al 1962) (Dalmasso, A.P. et al 1962 a) (Good, R.A. et al 1962) and neonatally thymectomized rats (Rieke, W. 1966) were found to be less efficient in inducing graft-versus-host reactions than equivalent numbers of cells from sham-thymectomized animals.

In general, delayed hypersensitivity reactions are reduced in intensity following neonatal thymectomy, In rats, thymectomized at birth, and tested in adult life, the ability to develop autoallergic encephalomyelitis was lost and the skin reaction to tuberculin was markedly reduced (Arnason, B.G. et al 1962 b). Delayed hypersensitivity reactions to bovine serum albumin were reduced by neonatal thymectomy (Arnason, B.G. et al 1962 a) (Jankovic, B.D. et al 1962) (Messini, M. et al 1964). In mice, thymectomy at birth protected the animals from the normally lethal meningeal inflammatory reaction following infection with lymphocytic choriomeningitis virus (Levey, R.H. et al 1963 b). Thymectomy in newly hatched chickens similarly reduced the intensity of delayed hypersensitivity reactions (Jankovic, B.D. and Isvaneski, M. 1963) (Jankovic, B.D. et al 1965 b).

In summary, neonatal thymectomy impairs those immune responses which depend upon T cells either for sensitization or the manifestation of the response.

THE EFFECTS OF ADULT THYMECTOMY

For many years it was thought that the thymus gland, whatever

its role, contributed little or nothing to the quality of health in the adult animal. This erroneous conclusion was based on two observations, firstly that adult thymectomy had no immediate effect on the physiology of the animal and secondly, that at puberty the thymus gland atrophies leaving only a very small stromal-epithelial structure. It is now known that the failure to detect physiological changes soon after adult thymectomy was due to the presence of long-lived, thymus-derived (T) cells that could function, in the absence of the thymus, for a considerable period of time. The life span of these T cells has been estimated to be over 100 days in rodents (Little, J.R. et al 1962) and perhaps several years in humans (Buckton, K.E. and Pike, M.C. 1964). The gradual depletion of T cells and the progressive loss of immune function that occurs following adult thymectomy went undiscovered by early investigators whose experimental observation period was usually not long enough to detect these changes. Thus, early experimenters found no difference in antibody titre when animals were tested a few weeks after thymectomy (Hammar, J.A. 1938) (Harris, T.N. et al 1948) (Maclean, L.D. et al 1957). Also, the mean survival time of allogeneic skin grafts was the same in rats (Heslop, J.H. and Nisbet, N.W. 1959) or mice (Miller, J.F.A.P. 1965 a) thymectomized or sham-thymectomized as adults.

In 1962 Miller observed that when (Ak x T6) F_1 mice were thymectomized at 12 weeks of age and given a single dose of 350 rads whole-body irradiation two weeks later, the mice sustained an impaired ability to produce agglutinins to sheep erythrocytes and to reject allogeneic skin grafts when challenged four weeks after irradiation (Miller, J.F.A.P. 1962 a). Sham-operated, irradiated mice recovered full immunological competence three to four weeks after irradiation (Cross, M. et al 1964 b) (Miller, J.F.A.P. et al 1963). Similar results were obtained with mice challenged with skin allografts

(Goedbloed, J.F. and Vos, O. 1965) (Leonard, L. and McHutchison, G. 1965) or tumour grafts (Globerson, A. and Feldman, M. 1964) (Globerson, A. et al 1962). An impaired ability to reject skin homografts existed in hamsters thymectomized at one week of age and irradiated two weeks later (Roosa, R.A. et al 1965). Rats, thymectomized when adult and then irradiated showed a reduced antibody response when challenged with sheep erythrocytes (Csaba, G. et al 1966). Rabbits, thymectomized at three weeks of age, and then exposed to 450r total-body irradiation and to simultaneous immunization with Salmonella typhi and conalbumin produced normal quantities of anti-H agglutinins but showed a depressed antibody response to conalbumin compared to non-irradiated, thymectomized animals (Konda, S. and Harris, T.N. 1966).

A more complete and longer lasting depression of immune competence occured when mice, thymectomized in adult life, were given potentially lethal whole-body irradiation and haemopoietic cell therapy (Miller, J.F.A.P. et al 1963) (Tyan, M.L. et al 1963) (Miller, J.F.A.P. et al 1964 a) (Cross, M. et al 1964 b) (Davies, W.E.Jr. et al 1964) (Goedbloed, J.F. and Vos, O. 1965) (Simmons, R.L. et al 1965). This result was also found for rats (Aisenberg, A.C. and Wilkes, B. 1964).

Globerson and Feldman reported that 550r whole-body irradiation produced a rapid depletion of small lymphocytes in the blood and tissues (Globerson, A. and Feldman, M. 1964). Davies reported that the most striking difference between thymectomized and intact, irradiated mice was a deficiency of small lymphocytes in the peri-arteriolar regions of the spleen and in the interfollicular cortex of the lymph nodes in the thymus-deprived mice (Davies, A.J.S. 1969 b). Other studies have also shown that thymectomy preceeding irradiation

markedly inhibits the regeneration of the lymphoid system (Auerbach, R. 1963) (Miller, J.F.A.P. et al 1964 a).

These results have been interpretted as showing that the thymus is required in adult life for the regeneration of the lymphoid system and the animal's subsequent return to immunological competence following total-body irradiation.

One of the first reports of a decrease in blood leukocyte levels following adult thymectomy was given by Paton and Goodall (Paton, D.N. and Goodall, A. 1904). Later, Pappenheimer found a decreased blood lymphocyte count in thymectomized rats (Pappenheimer, A.M. 1914 a). More recently other investigators have reported decreased lymphocyte numbers following adult thymectomy (Reinhardt, W.O. and Yoffey, J.M. 1956) (Nakamoto, A. 1957 b) (Comsa, J. 1957) (Beiring, F. 1960) (Metcalf, D. 1960). It was thought that defects similar to those seen after combined thymectomy and irradiation may become evident in adult thymectomized animals after an extended period of observation. Several workers, observed adult thymectomized mice for up to 22 months after thymectomy. Metcalf thymectomized or sham-thymectomized six-week-old (AKR x C57BL) F_1 mice and challenged groups of mice at 1 week, 11 months and 18 months after operation with an intra-peritoneal dose of sheep erythrocytes (Metcalf, D. 1965). Haemagglutinin titres in mice challenged immediately after thymectomy did not differ from those of control mice. However, in mice tested 11 months after thymectomy, some lower titres were found particularly in the early 19S phase of the response. In mice tested 18 months after operation about half of the thymectomized mice produced no detectable haemagglutinins, the remainder producing titres within the normal range. Similarly, Miller thymectomized two to three month old CBA and (AK x T6) F_1 mice and injected them with sheep erythrocytes at intervals between 2 and 22 months

after operation (Miller, J.F.A.P. 1965 b). He found that when 9 months or more had elapsed between thymectomy and antigenic challenge, thymectomized mice had fewer plaque-forming-cells in their spleens than intact controls, Miller also showed that lymphoid cells from C3Hf/Bi mice thymectomized at 2 - 4 months, were as effective as cells from control mice in producing graft-versus-host disease in (C3Hf x C57BL) F_1 mice when collected up to 6 months after thymectomy. Lymphoid cells from thymectomized mice collected after 6 months had elapsed between operation and sacrifice showed a lower capacity for producing graft-versus-host reactions. Taylor found that the capacity of lymph node cells from CBA mice, thymectomized at 2 - 3 months, to produce graft-versus-host disease in (C57BL x CBA) F_1 mice decreased sharply after 25 weeks had elapsed between thymectomy and sacrifice (Taylor, R.B. 1965). Taylor also found signs of an impaired capacity to respond to bovine serum albumin from between 10 and 16 weeks after thymectomy. Jeejeebhoy reported that Sprague-Dawley rats, thymectomized at 12 weeks of age, showed no decrease in the ability to produce circulating antibodies to sheep erythrocytes and tetanus toxoid even when tested 270 days after thymectomy. However, from 120 days after thymectomy, allogeneic skin graft rejection times were increased to about twice that found in control animals (Jeejeebhoy, H.F. 1965). Dukor and Dietrich found that in adult thymectomized mice the in vivo PHA-induced transformation of lymph node cells was reduced provided that 11 months had elapsed after thymectomy (Dukor, P. and Dietrich, F.M. 1967).

The results of the delayed effect of adult thymectomy and those of neonatal thymectomy indicate that the thymus is responsible for the production and maintenance of a long-lived population of immunologically competent T lymphocytes. Immunological deficiency

occurs if this population:

(A) is not allowed to develop, for example the animal is neonatally thymectomized.

(B) is damaged and not allowed to recover by prior or simultaneous thymectomy.

(C) is allowed to decay naturally over a period of several months (years) after adult thymectomy.

Recently, with the development of sensitive in vitro assays, it has been possible to demonstrate much earlier effects following adult thymectomy. These changes include a decreased capacity of lymphoid cells from adult thymectomized mice to respond in vitro to PHA (Johnson, J.M. and Wilson, D.B. 1970). Adult thymectomy has been reported to depress the mixed lymphocyte reaction in the rat (Robson, L.C. and Schwarz, M.R. 1971) (Folch, H. and Waksman, B.H. 1972). Adult thymectomy was also reported to be followed within 3 - 4 weeks by a decrease in the number of θ-positive cells when assessed by cytotoxicity or immunofluorescence tests (Schlesinger, M. and Yron, I. 1970) (Bach, J-F. et al 1975 b). Within 2 - 4 weeks following adult thymectomy the number of suppressor T cells has been reported to be decreased in the mouse (Zatz, M.M. and Goldstein, A.L. 1973). Although adult thymectomy did not decrease the number of lymph node and spleen cells capable of forming rosettes with sheep erythrocytes the concentration of azathioprine needed to inhibit rosette formation drastically and suddenly increased 5 - 6 days after thymectomy (Bach, J-F. et al 1971 a). Further experiments investigated a "thymic activity", present in the serum of normal animals and in thymic extracts, which restored the high sensitivity of rosette-forming cells, from the spleens of adult thymectomized mice, to azathioprine and

anti-θ serum (Bach, J-F. 1973). This serum thymic activity was found to disappear rapidly from the serum following thymectomy with a half life of two hours (Bach, J-F. 1973). This represents the earliest change detected following thymectomy.

RESTORATION OF IMMUNE COMPETENCE IN THYMUS-DEPRIVED ANIMALS.

This section will review various experimental approaches to the problem of restoring immunological competence to thymus-deprived animals.

I. Transfusion of lymphoid cells.

The rationale behind this approach is that neonatally thymectomized or adult thymectomized, irradiated animals have a deficiency of a line of immunologically competent T cells. This deficiency can be corrected, at least temporarily, by the transfusion of a suitable number of histocompatible, immunologically competent lymphocytes.

Thus 5 x 10^6 syngeneic lymph node cells from adult donors injected into one-week-old neonatally thymectomized mice prevented wasting in 60 - 80% of the recipient mice (Miller,J.F.A.P. 1964 b). The surviving mice were able to reject allogeneic skin grafts (Miller, J.F.A.P. 1963 a) (Miller, J.F.A.P. 1964 a) (Miller, J.F.A.P. 1964 b) Lymph node cells restored the capacity of neonatally thymectomized C3H mice to produce haemolysins to sheep erythrocytes (Trainin, N. et al 1965) and to restore the ability of neonatally thymectomized rats to produce antibodies and develop delayed hypersensitivity reactions to bovine serum albumin (Isakovic, K. and Waksman, B.H. 1965). Lymph node cells also restored the ability of adult thymectomized, irradiated mice to reject allogeneic skin grafts (Goedbloed, J.F. and Vos, O. 1965).

Spleen cells from non-thymectomized donors were effective in preventing the occurrence of the wasting disease and restoring immunological mechanisms in immune-deprived mice (Dalmasso, A.P. et al 1963) (Miller, J.F.A.P. 1964 a) (Miller, J.F.A.P. 1964 b) (Parrott, D.M.V. and East, J. 1964 b) (Yunis, E.J. et al 1964) (East, J. and Parrott, D.M.V. 1964) (Duplan, J-F. 1965). However, spleen cells from mice, thymectomized at birth, were ineffective in restoring immunological competence (Cross, M. et al 1964 b) (Miller, J.F.A.P. et al 1964 a) (East, J. and Parrott, D.M.V. 1964). Discriminant spleen assays performed using cells from neonatally thymectomized mice reconstituted with spleen cells showed that the restoration of immunological capacity was due to donor spleen cells (Dalmasso, A.P. et al 1963).

Injections of foetal liver cells (Cross, M. et al 1964 b) (Duplan, J-F. 1965) or bone marrow cells (Miller, J.F.A.P. 1964 a) (Cross, M. et al 1964 b) in doses up to 40×10^6 cells per mouse failed to reconstitute thymectomized irradiated mice. Those mice injected with foetal liver cells appeared to have greater immunological defects than those mice injected with adult bone marrow cells (Barnes, D.W.H. et al 1965). This was probably because foetal liver lacks the few immunologically competent cells that may be present in adult bone marrow. Indeed, in adult mice, thymectomized, lethally irradiated and injected with adult allogeneic bone marrow cells some deaths occurred from the development of "secondary disease", a syndrome similar to that seen in graft-versus-host reactions (Simmons, R.L. et al 1965). Therefore some cells in the adult bone marrow are competent to mount graft-versus-host reactions or are capable of maturing in the absence of the thymus to become immunologically competent.

A transfusion of dissociated thymic cells was found to be much less effective, on a cell to cell basis, than cells from the spleen and lymph nodes in correcting the immunological defects in immune-deprived mice (Miller, J.F.A.P. et al 1962) (Dalmasso, A.P. et al 1963) (Parrott, D.M.V. and East, J. 1964 a) (Miller, J.F.A.P. 1964 a) (East, J. and Parrott, D.M.V. 1964) (Trainin, N. et al 1965). This result indicated that only a minor population of cells within the thymus had obtained immunological competence or could obtain competence in the absence of the thymus.

Although immunological competence can be restored by the transfusion of immunologically competent lymphocytes this restoration is not permanent and immunological competence would be expected to decline in a manner similar to that seen following adult thymectomy.

II. Thymus transplantation.

Immunological functions have been restored in thymus-deprived animals to varying degrees by syngeneic, allogeneic and xenogeneic thymus grafts. The experimental results involving thymus transplantation in the analysis of the mechanism of immunological reconstitution are discussed in this section.

(A) Animal studies.

Thymus transplantation has been attempted for various reasons by many workers since the beginning of the century. Early workers reported that the grafts were quickly absorbed (Dudgeon, L.S. and Russell, A.E; 1905) (Hart, C. and Nordman, O. 1910). Renton performed intra-peritoneal auto and homotransplantation of the thymus in very young rabbits and guinea-pigs. He reported that all his autotransplants took and that in two out of six homotransplants healthy tissue was found 53 days after transplantation (Renton, J.M.

1916). Hoskins reported that in the tadpole thymus grafting did not effect the size or structure of the normal thymus, spleen or any other organ. The grafts grew well in every case (Hoskins, M.N. 1921). Demel reported such physiological effects as an increased growth of the long bones after the transplantation of rat thymus from donors aged 3 weeks, 2 months and 8 months respectively into young rats (Demel, R. 1922). Gottesman and Jaffe studied the regeneration of rat thymus autografts placed in the muscle of the abdominal wall (Gottesman, J.M. and Jaffe, H.L. 1926). In a series of 212 transplants removed from 53 albino rats aged between 38 and 45 days the following conclusions were drawn. Destructive changes take place during the first 48 hours, the most pronounced cellular disintegration taking place in the centre of the graft. Regenerative changes then occur which are usually complete by the 14th day when the newly formed thymic lobes show differentiation into cortex and medulla. Einhorn and Rowntree reported that frequent homologous thymus implants into normal parent (Einhorn, N.H. and Rowntree, L.G. 1938) and thymectomized parent (Einhorn, N.H. 1938) rats resulted in an accruing accelleration in the rate of growth and development in succeeding generations compared to control animals. More recently, thymus transplantation has been employed in obtaining a better understanding of the development of lymphoid leukaemia (Metcalf, D. 1966). These latter results are not discussed in this thesis.

There are many experimental variables to study in the analysis of the mechanism of the reconstitution of immune competence by thymus transplantation. For example, the ages of the donor and recipient, histocompatibility differences between donor and recipient, pretreatment (if any) of the thymus, site of placement of the graft,

size of the graft, treatment of the recipient, removal of the graft etc. etc.

Various sites for the placement of thymus grafts have been used, the most common being in the subcutaneous tissue and under the kidney capsule. Little difference in immunological recovery was detected between mice grafted at these two sites, except that the subcapsular site gave a less variable result (Leuchars, E. et al 1966). Metcalf found that thymus grafts placed under the renal capsule showed a 3 - 6 times greater growth rate compared with grafts placed subcutaneously (Metcalf, D. et al 1965). Similarly situated spleen grafts exhibited a 3 - 5 times more rapid growth than those in the subcutaneous tissues (Metcalf, D. and Bradley, R. 1965). It therefore appears that the subcapsular site is more efficient, probably by allowing a more rapid revascularization of the implant and thus a shorter period of ischaemia.

Unlike the growth of spleen grafts which is much faster if the host has been splenectomized (Metcalf, D. and Bradley, R. 1965) thymus grafts show remarkable autonomy of behaviour. Metcalf showed that the growth of thymus grafts was independent of either the state of the recipient's thymus and lymphoid tissue or the presence of other thymus grafts (Metcalf, D. 1963). The size attained by the individual graft fragment was, however, dependent upon both the age of the donor and the amount of medullary tissue present in the original fragment. Indeed, up to 40 thymus fragments have been implanted into single mice with no undue effect, most of the grafts regenerated and no hyperthymic effect was detected (Metcalf, D. 1966).

Studies on the histological and cytological regeneration of thymus grafts have been important in attempts to understand the mechanism of action of the normal thymus. A detailed account of the

regeneration of thymus grafts was published in 1965 by Dukor and associates (Dukor, P. et al 1965). The following sequence of events occurred after the implantation of thymus tissue either under the kidney capsule or subcutaneously in normal syngeneic hosts. Twenty four hours after grafting, all the implants showed haemorrhage and extensive necrosis in the centre and a rim of viable tissue in the periphery. The central necrosis was probably the result of an inadequate blood supply in the early hours after grafting. The viable rim contained both lymphoid and epithelial-reticular cells. Four days after transplantation the central area had been largely cleared of debris, vascular channels had appeared and many epithelial-reticular cells were in mitosis. There was extensive mitotic activity in the lymphoid rim. By day 5, a central core of epithelial elements surrounded by the vestiges of lymphocytic islands was present. After 8 days normal thymus architecture, with characteristic cortico-medullary lobes had become evident.

The extent of host contribution to the regeneration of thymus grafts was determined by using (C57BL x T6) F_1 host mice bearing a parental thymus graft. At metaphase, host cells could be recognised by the presence of the marker T6 chromosome. For about the first two weeks after grafting, the cells dividing in the graft were entirely derived from the donor cells surviving in the periphery of the original implant. During the 2nd to 3rd week, host cells were found dividing in the graft and by three weeks the dividing donor cell population had been entirely replaced by host cells. If such a graft was implanted into a new host then the original host cells were replaced by those of the new, second host (Harris, J.E. and Ford, C.E. 1964). Similar analyses by other workers have shown that the lymphoid

elements of thymus grafts are replaced by cells derived from the host. (Green, I. 1964) (Metcalf, D. and Wakonig-Vaartaja, R. 1964) (Schlesinger, M. and Hurvitz, D. 1968) (Cheers, C. et al 1972).

There is considerable evidence that thymic lymphocytes are continually replaced by stem cells entering the thymus. The thymus of animals recovering from lethal total-body irradiation followed by the administration of bone marrow or spleen cells is repopulated by cells derived from the inoculum (Ford, C.E. et al 1956) (Gengozian, N. et al 1957) (Popp, R.A. 1961)(Ford, C.E. and Micklem, H.S. 1963) (Schlesinger, M. et al 1965) (Micklem, H.S. et al 1966). Similarly, repopulation of the unirradiated thymus eventually occurs in animals in which only the lower third of the body was irradiated prior to bone marrow injection (Ford, C.E. et al 1966). The thymus of mice (Harris, J.E. and Ford, C.E. 1964) or of chicken embryos (Moore, M.A.S. and Owen, J.J.T. 1967) joined by parabiosis contained cells derived from the partner. All such experiments on cell traffic, however, are open to the criticism that they are not representative of the normal situation, In the experiments described above the animals were subjected to abnormal conditions and stress and the results have to be viewed with this in mind.

There are basically two theories, neither of which is mutually exclusive, concerning the mechanism of thymus function. Firstly, the cellular theory: the thymus acts as a 'factory' converting incoming stem cells into immunologically competent lymphocytes. Release of these cells from the thymus maintains the pool of immunologically competent lymphocytes. Secondly, the humoral theory: the thymus secretes a factor (or factors), which, by itself or in combination with other events, converts immunologically incompetent cells into immunologically competent lymphocytes.

The importance of thymus graft-derived cells in immune restoration was shown by the following. When neonatally thymectomized mice were reconstituted with a cytologically distinct thymus graft a few cells of thymus graft origin were found dividing in the spleen (Miller, J.F.A.P. 1962 b) (Miller, J.F.A.P. 1963 a). A similar finding was reported in adult thymectomized, reconstituted mice (Leuchars, E. et al 1965). These thymus graft-derived cells were found to divide in larger numbers than host cells after an injection of sheep erythrocytes (Leuchars, E. et al 1964) (Davies, A.J.S. et al 1966) (Miller, J.F.A.P. et al 1966). Doenhoff and co-workers analysed the fate of thymus graft-derived cells by studying the response to PHA in adult thymectomized, irradiated and reconstituted mice in which the thymus graft and bone marrow innoculum were chromosomally distinct (Doenhoff, M.J. et al 1970) (Doenhoff, M.J. and Davies, A.J.S. 1971). Chromosome analysis of the cells responding to PHA revealed that, at 120 days after irradiation 60% of blood lymphocytes, 30% of spleen lymphocytes and about 35% of lymph node cells were derived from the donor thymus. Even 450 days after grafting there was a considerable number (25%) of thymus donor cell mitoses in the peripheral blood. These results provide strong evidence for the importance of the cellular theory of thymus function. Further evidence must include a demonstration, under physiological conditions, of the release of lymphocytes from the thymus. This latter topic is not considered in this thesis.

The lymphopenia and the various immunological defects found in neonatally thymectomized mice have been corrected by syngeneic, normal thymus grafts (Miller, J.F.A.P. 1961) (Miller, J.F.A.P. 1962 c) (Dalmasso, A.P. et al 1963) (East, J. and Parrott, D.M.V. 1964)

(Miller, J.F.A.P. 1964 b) (Schaller, J.T. and Stevenson, J.K. 1965) (Stutman, O. et al 1969). Adult thymectomized, irradiated mice have similarly been restored (Globerson, A. and Feldman, M. 1964) (Miller, J.F.A.P. et al 1964 a) (Dukor, P. et al 1965) (Leuchars, E. et al 1965). Nude mice, a strain with congenital thymic aplasia (Pantelouris, E.M. 1968) have also been restored by thymus grafting (DeSousa, M.A. and Pritchard, H. 1974).

The following section deals with those aspects of immune restoration by thymus grafts that suggest the involvement of humoral factors. Short exposures (7 - 10 days) of neonatally thymectomized mice to a syngeneic thymus graft produced some restoration of immune capacity (Cross, M. et al 1964 a) (Miller, J.F.A.P. et al 1966) (Stutman, O. et al 1971). Neonatally thymectomized mice bearing, for one or two weeks, thymuses that had been irradiated in vitro with 500r showed no thymus donor-derived cells in the lymphoid system even in those mice capable of responding to antigenic stimuli (Miller, J.F.A.P. et al 1966). It was concluded that the restoration was dependent upon the integrity of thymus epithelial-reticular cells. Such restoration was found however, to be not self sustaining and declined progressively with age (Stutman, O. et al 1971).

When an allogeneic thymus is implanted into normal or irradiated hosts, the graft is infiltrated by lymphocytes and histiocytes and is eventually rejected (Miller, J.F.A.P. and Osoba,D. 1967). A similar sequence of events occurred when allogeneic thymus grafts were implanted into adult thymectomized irradiated and bone marrow protected mice (Dukor, P. et al 1965). The grafts failed to become lymphoidal, never showed the presence of proliferating donor-type cells and were eventually rejected. The thymectomized irradiated mice grafted with

such thymuses, however, showed partial recovery of immunological cppabilities and were able to reject skin of thymus donor type with an accelerated second-set response (Leuchars, E. et al 1965). These results were interpreted as evidence for a non strain-specific humoral maturation of bone marrow cells that was dependent upon the existence of surviving epithelial-reticular cells in the thymus graft (Miller, J.F.A.P. and Osoba, D. 1967).

Allogeneic thymus tissue implanted into neonatally thymectomized mice behaved more like syngeneic transplants in striking contrast to their behaviour in adult, thymectomized, irradiated mice. Thus, the implants regained normal architecture by proliferation of donor elements and were not rejected. In many cases these animals were specifically immunologically tolerant of thymus donor type skin (Miller, J.F.A.P. 1962 c) (Dalmasso, A.P. et al 1963) (Stutman, O. et al 1969 a) yet had the ability to reject third party allogeneic skin grafts. Mice of several strains, if thymectomized within 24 hours after birth, develop a wasting disease a few weeks later. By implantation of multiple allogeneic thymus grafts it was possible to prevent the development of this syndrome and to repair the immunological capacity of these animals to reject skin homografts (Schaller, R.A. and Stevenson, J.K. 1965). Thymus grafts across non-H_2 histocompatibility differences were usually successful in reconstituting a significant percentage of neonatally thymectomized mice and the success of such grafting varied with the strains of mice used (Stutman, O. et al 1969 a). Allogeneic grafts across major H_2 histocompatibility barriers often failed to achieve immunological restoration and frequently induced a severe and often fatal graft-versus-host reaction (Stutman, O. et al 1968 b) (Stutman, O. et al 1969 a). Discriminant spleen assays always showed an important host component and inferred that host cell maturation had

occurred (Stutman, O. et al 1969 a). The differing degrees of restoration using thymus grafts from allogeneic donors is possibly related to decreased cellular traffic to the thymus when donor and host differed across H_2 histocompatibility barriers (Stutman, O. and Good, R.A. 1969).

Reconstitutive success with xenogeneic thymus grafts has been reported. Hamster or rat thymus, when grafted subcutaneously into neonatally thymectomized mice allowed 60 - 70% of the mice to reject skin grafts and to produce normal haemolysin titres (Law, L. 1966 b). Peripheral blood lymphocyte counts were also partially restored. Few lymphoid cells were found in the grafts after ten days, however, epithelial-reticular cells survived in many grafts for up to 30 days at which time the grafts were considered rejected (Law, L. 1966 b). Neonatally thymectomized C57BL mice were incapable of rejecting a Walker 256 carcinoma which killed untreated thymectomized controls. The implantation of a thymus from Sprague-Dawley rats allowed the mice to reject the carcinoma (Hallenbeck, G.A. et al 1969). Not all cases of xenogeneic thymus transplantation brought about immunological recovery. Yunis and co-workers, for example, grafted neonatally thymectomized C3H mice with a newborn Holtzman rat thymus and observed no immunological restoration despite histological evidence showing that most of the grafts survived transplantation (Yunis, E.J. et al 1964 b).

A series of experiments with a non-lymphoid thymoma emphasized the importance of the non-lymphoid elements in thymus graft restoration (Stutman, O. et al 1968 a). The tumour was one of several that arose after direct intrathymic application of dimethylbenzathracene (DMBA) into newborn mice. When this thymoma was grafted into neonatally

thymectomized syngeneic or allogeneic mice an immunologically restoring effect was noted. Discriminant graft-versus-host analysis in the allogeneic combination showed that all demonstrable immunologically competent cells in the spleens of the restored animals were of host origin (Stutman, O. et al 1968 a).

Histological studies have suggested that epithelial-reticular cells have an important role in controlling lymphopoiesis within the thymus. In mice (Metcalf, D. 1966) and guinea-pigs (Mandel, T. 1969) epithelial cells were found to be associated with greater numbers of mitotic lymphoid cells than due to statistical associations. Hays, who has studied the transplantation of lymphocyte-depleted thymus grafts, has suggested that epithelial cells effect lymphoid cells only within the thymus. Neonatal thymuses were placed in millipore diffusion chambers and implanted intra-peritoneally into adult mice. Eight days later, when the thymuses were recovered, they consisted of an epithelial-reticular remnant containing no lymphocytes. Long term grafts of these remnants into neonatally thymectomized mice or adult, thymectomized, irradiated mice restored immunological competence and allowed partial lymphocyte recuperation (Hays, E.F. 1967) (Hays, E.F. 1969). If, however these grafts were removed after 7 or 14 days, then little recovery of immunological competence was observed (Hays, E.F. and Alpert, P.F. 1969). Hays believed that epithelial-reticular cells function within the thymus acting on precursor cells that eventually leave the thymus to populate the lymphoid tissues.

It has been repeatedly suggested, especially from studies involving allogeneic and xenogeneic thymus grafts, that the presence of epithelial-reticular cells within the graft was essential for immunological recovery. It has therefore been postulated that

immunological recovery involves host cell maturation under the influence of a humoral factor liberated from the epithelial-reticular cells of the thymus (Miller, J.F.A.P. 1964 c) (Schaller, R.A. and Stevenson, J.K. 1965) (Law, L. 1966 b) (Stutman, O. et al 1968 a) (Hallenbeck, G.A. et al 1969). It has not been possible, however, to show conclusively, by the techniques described in this section whether such a factor exists, or, if it exists whether it is active at a distance from the thymus or whether it functions only within the microenvironment of the thymus.

(B) Human studies.

Thymus transplantation in man has been attempted, often in desperation (Marcolongo, R. and Paolo, N. Di. 1973) (Rzepecki, W.M. et al 1973) (Serrou, B. 1973) usually with the aim of trying to increase immunological performance in critically ill patients. The majority of clinical reports concerning thymus transplantation are of little value in attempts to understand the function of the thymus. This is due mainly to complication by other treatment and lack of controls. Most success, that is clinical improvement, involving thymus transplants have been obtained with patients suffering from a congenital failure of the development of the thymus-dependent lymphoid system. The best documented lesion of this type is the DiGeorge syndrome (Park, B.H. and Good, R.A. 1974). The apparent etiology of the DiGeorge syndrome is a failure of development of the epithelial anlagen derived from the third and fourth pharyngeal pouches. These anlagen, after full differentiation, develop into the thymus, parathyroids and probably those clear cells of the thyroid responsible for calcitonin production. This abnormality has not shown a familial incidence and

does not appear to be heriditary, All infants with this syndrome have neonatal tetany. The hypocalcaemia tends to ameliorate with age during the first year of life. Hypertelorism, a shortened lip philtrum, low-set ears, notched pinnae and nasal clefts are some of the characteristic features. In addition abnormalities of the great blood vessels are usually present. Infants with thymic aplasia show greatly increased susceptibility to viral, fungal and bacterial infections, which may ultimately be overwhelming. Normal, or near normal, numbers of plasma cells are present and serum concentrations of immunoglobulins are normal. A moderate to severe depletion of lymphocytes is seen in the thymus-dependent regions of the lymph nodes and spleen. Although antibody responses to some primary stimuli may be normal, delayed hypersensitivity is not manifested to the common skin test antigens. Sensitization to dinitrofluorobenzene (DNFB) is unsuccessful or yields a weakly positive result. Skin allograft rejection is delayed or absent. The peripheral blood lymphocytes respond poorly, or not at all, to PHA. The immunological characteristics of this syndrome therefore closely resemble those of the thymus-deprived rodent. Ideas for the treatment of infants with the DiGeorge syndrome were obtained from inspection of the experimental results pertaining to the immunological restoration of thymectomized rodents. These results indicated that the best chance of a permanent recovery of immune function lay in grafting whole, or pieces of thymus, rather than by injection of separated lymphocytes from the thymus or other organs. Animal models had shown that thymus grafts across major histocompatibility barriers often failed to achieve immunological reconstitution and furthermore frequently induced a severe and fatal graft-versus-host disease (Stutman, O. et al 1968 b) (Stutman, O 1969 a).

In order to minimise the chance of initiating a fatal graft-versus-host reaction foetal human thymuses have been used for transplantation since foetal thymuses contain less immunologically competent lymphocytes than neonatal or adult thymuses.

A summary of some of the better documented attempts at immunological restoration by thymus transplantation is shown in table 1.2. The first two reported cases (August, C.S. et al 1968) (Cleveland, W.W. et al 1968) demonstrated that an apparently full restoration of immune function in children with DiGeorge syndrome followed shortly after the transplantation of embryonic thymus tissue. August showed that the PHA response became normal four days after grafting and that the cells responding to PHA were of host origin (August, C.S. et al 1968). A similarly prompt restoration of PHA response was reported by Cleveland, (Cleveland, W.W. et al 1968). Early host cell maturation was also reported by Steele who found that the PHA response became normal eight days after grafting a thymus enclosed within a millipore diffusion chamber (Steele, R.W. et al 1972). A six year old boy with severe T cell deficiency, having none of the features common to the DiGeorge syndrome, became immunologically normal by several criteria, on the second day after thymus transplantation when no cells of graft origin could be detected (Foroozanfar, N. et al 1975). These four cases in which immunological restoration occurred within several days and was due to the maturation of host cells, provide persuasive evidence that the thymus provided some critical factor, probably humoral, that converted immunologically incompetent lymphocytes into competent lymphocytes. Other experience (Gatti, R.A. et al 1972) (Ammann, A.J. et al 1973) (Kazimiera, J. et al 1973) showed that although immunological restoration occurred it

did so progressively over several months. The mechanism of restoration probably involved thymus processing of host precursor cells. It is not known why in some cases immunological competence was restored promptly whilst in others several months of thymic influence was necessary.

Doubts concerning the capacity of foetal thymus transplants to restore immune function in the treatment of DiGeorge syndrome (Dempster, W.J. 1969) have been partly dispelled. Experiments done by Biggar, Stutman and Good have shown that, in an animal model, an embryonic mouse thymus which was not lymphoidal, could restore immune competence in syngeneic and allogeneic systems (Biggar, W.D. et al 1972). Human foetal thymuses have also been shown to survive transplantation (Kay, H.E.M. 1969) (Hong, R. et al 1972). Other doubts as to the effect of the thymus graft occur because of the uncertain immunological condition of the patient. The documentation of cases involving T cell deficiency has not yet reached the stage where the lesions are fully recognised and a variety of different conditions appear to exist. For example, it is quite common to find small pieces of histologically normal thymus tissue at necropsy in some patients with DiGeorge syndrome (Lischner, H.W. and Good, R.A. 1969). Also, spontaneous recovery of cellular immune responses has been observed in a patient with DiGeorge syndrome (Gatti, R.A. et al 1972).

Table 1.2 A summary of results of thymus transplantation in humans.

Year	Authors	Subject	Thymus graft procedure	Immunological responses	
				Before graft	After graft
1968	August, C.S. et al (1968).	21 month old male, DG_1 syndrome	Implantation of thymus fragments from 16-week-old female foetus.	Defective allograft rejection No delayed hypersensitivity to monilia antigen and $DNFB_2$ No PHA response.	Prompt and long lasting restoration of responses. PHA response normal by 4 days.
1968	Cleveland, W.W. et al (1968).	7 month old male, DG syndrome.	Implantation of thymus from 13-week-old female foetus.	Lymphopenia. No PHA response.	PHA response normal 3 weeks after graft. Lymphocyte count normal within 1 - 2 days.
1972	Steele, R.W. et al (1972).	10 week-old female, DG syndrome.	Implantation of thymus from 13-week-old female foetus within a MDC.	No delayed hypersensitivity to common skin test antigens. Negligible PHA response.	Increased PHA response 6 hours after graft, normal PHA response 8 days after graft.
1972	Gatti, R.A. et al (1972).	45 day old male, 'DG variant'.	Implantation of thymus from 10-12 week old male foetus.	PHA and PWM_3 response absent, MLR_5 normal. Low or absent Ig levels.	Normal PHA and PWM response 3 months after graft. Ig levels normal. Rejection of 2 skin grafts. Normal numbers of lymphocytes and plasma cells.
1972	Hong, R. et al (1972).	2 year old child with cartilage-hair hypoplasia, with deficient CMI_4. Progressive vaccinia.	Implantation of 3 foetal thymuses (aged 12, 18 and 14 weeks) over a 4 month period.	Lymphopenia. No cutaneous response to skin test antigens. No in vitro reactivity to vaccinia antigen, allogeneic cells or PHA.	Slight increase in lymphocyte numbers. Slight response to PHA and allogeneic cells.
1973	Ammann, A.J. et al (1973).	4 week old infant with sex-linked combined immunodeficiency	Implantation i.p. of 14 week old foetal thymus.	Lymphopenia. Poor PHA response	Total lymphocyte count normal after 2 months. 9 months after graft PHA response normal.

Table 1.2 continued

Year	Authors	Subject	Thymus graft procedure	Immunological responses	
				Before graft	After graft
1973	Kazimiera, J. et al (1973).	Infant with DG syndrome.	Foetal thymus transplant.	No PHA response. No response to skin test antigens. Decreased number of T cells, increased B cells.	Progressive increase in PHA and skin test response. A fall in number of B cells.
1974	Jose, D.G. et al (1974).	2 year old male with thymic aplasia	4 foetal thymus "cell" grafts.	No delayed responses to skin test antigens. PHA, MLR responses negligible. No sheep erythrocyte rosette-forming-cells.	Temporary restoration only.
1975	Foroozanfar, N. et al (1975).	6 year old male with severe T cell deficiency (not DG syndrome).	Implantation of 20 week old foetal thymus.	Subnormal in vitro response to PHA and candida antigen MLR reduced. Reduced number of cells forming rosettes with sheep erythrocytes.	On second day after grafting PHA, MLR and response to candida were normal and remained normal.

Abbreviations:

1. DG DiGeorge syndrome
2. DNFB Dinitrofluorobenzine.
3. PWM Pokeweed mitogen.
4. CMI Cell-mediated immunity.
5. MLR Mixed lymphocyte reaction.
6. MDC Millipore diffusion chamber.

III Thymus in millipore diffusion chambers

Neonatal thymectomy in rodents leads to the development of several syndromes as outlined in previous sections of this chapter. A syngeneic thymus implant was found to be effective in preventing the occurrence of these syndromes. A logical next step was to enclose a thymus graft in a cell-impermeable chamber that allowed the passage of nutrients but prevented cells from entering or leaving the chamber. The implantation of these chambers into thymus-deprived rodents contributed to an evaluation of the role of thymic humoral factors, in thymus graft restoration.

Basically two types of container for thymus grafts have been used: chambers and envelopes. Diffusion chambers were prepared from rings of plastic, such as plexiglass or lucite, of various diameters, to which nitrocellulose or cellulose ester membranes were attached by means of a solution of lucite in acetone or by some other cement such as acryloid glue (Algire, G.H. et al 1954) (Nettesheim, P. et al 1966). Envelopes were prepared either by folding a piece of filter membrane or by approximating two equal areas of this material, the open edges being sealed with a thin film of acetone. Millipore filters of various mean pore sizes have been used for the construction of such chambers and envelopes.

In the evaluation of the results it is crucial to know whether the diffusion chambers and envelopes were, in fact, impermeable to cells. Cell leakage could occur either through pores of excessive size or due to faulty contruction of the chamber. Chambers have generally been found more reliable than envelopes but even their construction requires a certain skill and experience. Nettesheim found that 70% of chambers constructed by inexperienced operators

failed to be cell-impermeable (Nettesheim, P. et al 1966). The filters are very fragile and can easily be damaged, particularly at the junction points of filter and lucite ring (Davies, A.J.S. 1969 a). Envelopes are particularly difficult to handle without producing visible ruptures since the acetone sealed filters become extremely brittle (Stutman, O. et al 1969 b).

Various estimates of cell permeability have been published. Osoba found that when a block of solid Gross leukaemia tissue, enclosed within a chamber of mean pore size 0.1μ, was implanted into the peritoneal cavities of three-week-old mice, 2 out of 20 mice developed leukaemia (Osoba, D. 1965 a). All 10 control mice given an implant of leukaemia tissue in an open diffusion chamber died within three weeks of implantation. Barclay investigated the cell-permeability of chambers of pore size 3.0μ, 0.45μ, 0.1μ and 0.01μ (Barclay, T.J. 1964). He enclosed a lymphomatous ascites tumour in the chamber and placed the chamber in the peritoneal cavity of (BALB/c x C57BL) F_1 mice. Chambers of pore size 3.0μ and 0.45μ were considered cell-permeable as judged by significant deaths in mice bearing these filters. Law and co-workers using chambers constructed with millipore filters, of 0.45μ pore size found that 6 out of 16 mice and 4 out of 16 mice respectively succumbed when a solid reticulum cell sarcoma (L - 10431) and a lymphocytic neoplasm (L - 1210) were enclosed in these chambers (Law, L.W. et al 1964 b). All mice inoculated intraperitoneally with either neoplasm, not enclosed within a chamber, died. In half of the mice that died from tumour enclosed within the chamber a disrupted chamber was found and the remaining 5 deaths were considered as probably representing leaks. Stutman and co-workers implanted T6 marked thymus cells enclosed in diffusion chambers into chromosomally unmarked

mice in order to investigate the passage of cells through membranes of various pore sizes (Stutman, O. et al 1969 b). They found that cells in chambers prepared with filters of 0.45μ and 0.30μ pore size were able to escape and divide in the host tissues, whereas chambers constructed with 0.22μ or 0.10μ filters did not permit the passage of cells. Chambers made from filters of 0.45μ and 0.30μ pore size and implanted empty, were subsequently found to contain host cells. Stutman also showed that the percentage of host restoration declined as the pore size decreased. This result correlated with better survival of tissue in the chambers of larger pore size. Spleen fragments enclosed in chambers of 0.45μ pore size produced a modest restoration of immune function in neonatally thymectomized mice.

On the basis of the above results it would seem that only chambers with pore sizes of 0.22μ or less could claim to approach the requirement of cell inpenetratability. As most of the results with millipore diffusion chambers (MDC'S) have been obtained with chambers of pore size 0.30μ or greater an unfortunately unknown percentage of the restoration was, almost certainly, due to thymus graft cells.

A survey of experiments using thymus-containing MDC's and envelopes is presented in table 1.3. In most cases immune deprivation of the recipients was achieved by neonatal thymectomy and in a few cases by adult thymectomy and whole-body irradiation. The age of the implanted thymus varied from embryonic (Osoba, D. and Miller, J.F.A.P. 1963).(Osoba, D. and Miller, J.F.A.P. 1964) to young adult (Stutman, O. et al 1969 b) or adult (Barclay, T.J. 1964); the most common age being from newborn to 14 days old. The genetic relationship of the donor and recipient was either syngeneic or allogeneic. All the implantations were performed intraperitoneally at times depending upon the purpose

of the experiment. When the experiment was designed to show possible prevention of the consequences of neonatal thymectomy the chambers were implanted up to three weeks after thymectomy. When reversal of the post-thymectomy wasting syndrome was attempted then implantation was performed after the onset of wasting, some 6 - 8 weeks after neonatal thymectomy (Schaller, R.T. and Stevenson, J.K. 1967) (Stutman, O. et al 1969 c).

Following the implantation of thymus tissue enclosed within a MDC into a neonatally thymectomized mouse most of the consequences of thymic deprivation did not occur or occurred to a lesser degree. The high incidence of wasting characterized by progressive weight loss, diarrhoea, ruffling of the fur, hunching and eventual death, which was the normal occurrence after neonatal thymectomy, was prevented (Levey, R.H. et al 1963 a) (Osoba, D. and Miller, J.F.A.P. 1963) (Osoba, D. and Miller, J.F.A.P. 1964) (Osoba, D. 1965 a) (Stutman, O. et al 1969 b). Restoration of lymphocyte numbers in the blood and lymphoid organs did not occur consistently. Several authors described marked restorative effects on circulating lymphocytes and lymphoid tissue cellularity (Levey, R.H. et al 1963 a) (Law, L.W. et al 1964 b) (Osoba, D. and Miller, J.F.A.P. 1964) (Law, L.W. 1966 a) (Trench, C. et al 1966). However, other authors reported little or no increase in lymphocyte levels accompanying restoration of immune responses (Osoba, D. and Miller, J.F.A.P. 1963) (Barclay, T.J. 1964) (Osoba, D. 1965 a) (Aisenberg, A.C. and Wilkes, B. 1965) (Wong, F.M. et al 1966) (Biggart, J.D. 1966 a) (Sherman, J.D. 1967 a) (Stutman, O. et al 1969 b). It is now apparent, in the light of current knowledge of the thymus-dependent areas of the lymphoid tissue, that some of the photographs published showing recovery of lymphocyte numbers indicated that the

treatment had very little influence on thymus-dependent areas in the lymph nodes and Peyers patches and that lymphoid proliferation occurred mainly in the thymus-independent areas.

Neonatally thymectomized mice manifest an impaired ability to respond to sheep erythrocytes as measured by decreased circulating antibodies (Fahey, J.L. et al 1965) and a reduced intensity of delayed hypersensitivity reactions (Stutman, O. et al 1969 b). Normal or near normal haemagglutinin titres to sheep erythrocytes were obtained when neonatally thymectomized CBA or (CBA x T6) F_1 mice were implanted with envelopes containing syngeneic or allogeneic, embryonic or newborn thymuses (Osoba, D. and Miller, J.F.A.P. 1964) (Osoba, D. 1965 a). Similarly 7 out of 16 neonatally thymectomized rats grafted with a thymus enclosed in a MDC showed restored haemagglutination responses to sheep erythrocytes (Biggart, J.D. 1966 b). When neonatally thymectomized C3H and DBA mice were grafted with thymus contained in diffusion chambers the haemolysin (19S) response to sheep erythrocytes was restored. The pattern of response and mean titre were similar to that of neonatally thymectomized mice bearing subcutaneous grafts of syngeneic thymus (Law, L.W. et al 1964 a). Adult, thymectomized, CBA mice subjected to 950r total-body irradiation and bone marrow therapy were very poor responders to sheep erythrocytes, measured by the number of plaque-forming cells in the spleen six days after challenge (Osoba, D. 1968). When these mice were grafted with a millipore envelope containing two lobes of thymus from a newborn syngeneic donor a significantly higher plaque-forming cell response was obtained in comparison to untreated thymectomized mice or to thymectomized mice containing lymph nodes or spleen in the envelope. Neonatally thymectomized mice bearing thymus-containing chambers were also found to be restored on the basis of

of their ability to react positively in the production of delayed hypersensitivity reactions in response to footpad injection of sheep erythrocytes (Stutman, O. et al 1969 b).

It was found that when neonatally thymectomized mice (Osoba, D. and Miller, J.F.A.P. 1963) (Osoba, D. and Miller, J.F.A.P. 1964) (Osoba, D. 1965 a) (Schaller, R.T. and Stevenson, J.K. 1967) (Stutman, O. et al 1969 b); neonatally thymectomized rats (MacGillivray, M.H. et al 1964) (Aisenberg, A.C. et al 1965) and adult thymectomized, whole-body irradiated mice (Barclay, T.J. 1964) (Miller, J.F.A.P. et al 1964 a) were tested with skin allografts those animals bearing thymus-containing chambers showed a significantly reduced time to rejection compared with those animals not so grafted. Also when 14 day old thymus glands, enclosed in a MDC, were implanted into neonatally thymectomized C57BL/6 mice, the ability to reject a rat Walker carcinoma was restored (Hallenbeck, G.A. et al 1969). In a study of the oncogenic effect of polyoma virus it was found that thymectomy at three days of age, in several inbred strains of mice, resulted in a marked increase in the frequency of neoplasms following infection with polyoma virus. However, when thymectomized mice were grafted with a neonatal thymus in a MDC then these mice became much more resistant to the oncogenic threat imposed by the virus (Law, L.W. and Ting, R.C. 1965). Normal, unoperated Swiss mice inoculated intracerebrally with lymphocytic choriomeningitis virus (LCM) showed a 100% mortality within 8 days after virus infection. Neonatally thymectomized mice, with or without empty diffusion chambers, incurred no deaths from the virus by 14 days (Levey, R.H. et al 1963 b). This has been attributed to a depression of some type of tissue hyperreactivity involving lymphoid cell competence (Rowe, W.P. et al 1963). This competence was restored in about half of the

thymectomized mice by a graft of a thymus contained in a MDC as judged by death from the virus (Levey, R.H. et al 1963 b). The inability of spleen cells from neonatally thymectomized C3Hj/Bi mice to induce a graft-versus-host reaction upon injection into 8-day-old normal (C3H x C57BL/1)F_1 recipients was overcome by prior implantation of the thymectomized donors with MDC's containing thymus tissue (Stutman, O. et al 1969 b). When neonatally thymectomized C3H/HeJ mice were restored by chambers containing syngeneic or allogeneic thymuses it was found that the restoration was due to host cell maturation as judged by a discriminant spleen assay (Schaller, R.T. and Stevenson, J.K. 1967).

Most of the experimental work has been done using mice, other species however have produced similar results. For example, neonatal thymectomy in the rat leads to an impaired capacity for delayed hypersensitivity responses to bovine serum albumin (BSA) (Jankovic, B.D. et al 1962) (MacGillivray, M.H. et al 1964) (Aisenberg, A.C. and Wilkes, B. 1965). Implantation of millipore chambers containing thymus led to a significantly restored response (MacGillivray, M.H. et al 1964) and a partially restored response (Aisenberg, A.C. and Wilkes, B. 1965) measured by the ability to produce delayed hypersensitivity reactions to BSA. A partial restoration of circulating antibody formation in response to BSA was also noted (MacGillivray, M.H. et al 1964). Golden hamsters, when thymectomized at 12 - 14 days of age, were found to show a depressed antibody response when challenged with human gamma globulin (HGG). If such thymectomized hamsters were implanted with millipore envelopes containing a 2-week-old thymus then a normal antibody response to HGG was obtained (Wong, F.M. et al 1966). In rabbits a similar depression of the immunological response to HGG which accompanied

thymectomy was restored by autotransplants of thymus in a MDC Trench, C. 1966).

The experimental results suggest that thymus tissue enclosed in cell-impermeable millipore chambers can prevent most of the effects of immune deprivation. It is important to consider the specificity of this claim. For example, a marked adjuvant effect of diffusion chambers on the antigenicity of two soluble antigens, haemocyanin and ferritin has been reported (Adler, F.L. and Fishman, M. 1962). However, in the experiments reported here, empty chambers implanted into test animals produced no observed restoration of the responses studied. Also, when a wide variety of other tissues were enclosed in the chamber it was found that restoration of immune function was dependent upon the presence of thymus tissue (Wong, F.M. et al 1966) (Osoba, D. 1965 a) (Osoba, D. 1968) (Stutman, O. et al 1969 b). A modest restoration of immune function was reported, however when spleen tissue was enclosed in chambers of 0.45μ mean pore size (Stutman, O. et al 1969 b). It was probable that in the latter case restoration was due to spleen cells escaping from the chamber. Experiments involving the bursa of Fabricius are relevant here. It has been reported that bursas enclosed in MDC's of pore sizes 0.45μ and 0.10μ induced immunological recovery when implanted into bursectomized chickens, measured by increased antibody responses (Pierre, R. L. St. and Ackerman. 1965) (Jankovic, B.D. and Leskowitz, S. 1965). Dent repeated these experiments and reported that the effect of the bursa-containing chambers could be related to bacterial contamination (Dent, P.B. et al 1968). He found a restorative effect with 5-day-old bursa and no effect with embryonic bursa. Bacterial cultures showed that the chambers containing bursas from

5-day-old chicks were contaminated whereas those containing embryonic tissue were not. Dent also showed a restorative effect using chambers containing chicken bowel. These experiments show that positive results should be interpreted cautiously.

At the end of the experiments the chamber was usually found encapsulated in a loose fibrous matrix without evidence of disruption. When the thymus remnants were examined several weeks after implantation they were found to consist mainly of epithelial-reticular elements and fibroblasts with no, or few, scattered nests of lymphocytes (Levey, R.H. et al 1963 a) (Osoba, D. and Miller, J.F.A.P. 1963) (Levey R.H. et al 1963 b) (Osoba, D. and Miller, J.F.A.P. 1964) (Osoba, D. 1965 a) (Trench, C. 1966) (Wong, F.M. et al 1966) (Weiss, L. and Miller, J.F.A.P. 1966). The lymphocytes in the thymus usually degenerated within the first two weeks after implantation (Osoba, D. and Miller, J.F.A.P. 1964) (Weiss, L. and Miller, J.F.A.P. 1966) (Hayes, E.F. 1967). It has therefore been suggested that the epithelial cells of the thymus are responsible for the production of a humoral factor capable of effecting immune restoration. The suggestion (Parrott, D.M.V. and East, J. 1964 a) that the breakdown products of lymphoid cells might be responsible for the observed restorative effects seems untenable for the following reasons. When the chambers were removed from hamsters after four weeks, the tissue remnants were found to be devoid of lymphocytes. These chambers, however, when reimplanted into thymus-deprived recipients exerted a similar effect to that obtained using fresh thymus in the chamber (Wong, F.M. et al 1965) (Wong, F.M. et al 1966). Non-lymphoid, functional thymomas, induced by neonatal intrathymic injections of dimethyl-benzanthracene have been found capable of restoring

thymectomized mice by intraperitoneal placement of diffusion chambers containing the thymoma (Stutman, O. et al 1969 b). Cytological indications of secretory activity in thymus epithelial cells enclosed in millipore chambers have also been obtained (Weiss, L. and Miller, J.F.A.P. 1966) (Shelton, E. 1966). It therefore seems most probable that the source of the hypothetical humoral factor is the thymus epithelial cell.

The diffusible factor from the thymus graft in some manner acts upon host cells to endow them with immune competence. Restoration was obtained using normal syngeneic or allogeneic thymuses. When the lymphoid organs of the reconstituted animals were examined the chromosome make-up found in mitotic cells was of host type (Osoba, D. and Miller, J.F.A.P. 1963) (Osoba, D. and Miller, J.F.A.P. 1964) (Stutman, O. et al 1969 b). The discriminant spleen assay has also shown that the donor thymus did not contribute with its cell population to the repair process (Schaller, R.T. and Stevenson, J.K. 1967). Little is known concerning the site of interaction of the target cell andthe thymic humoral factor. This interaction could occur at short range amongst the connective tissue surrounding the chamber and / or at a considerable distance from the chamber. Stutman and Good reported, in a preliminary study, that haematopoietic cells migrate to the connective tissue surrounding the diffusion chamber containing thymus tissue whereupon they acquire the ability to migrate to lymph nodes and to respond to antigenic stimulation (Stutman, O. and Good, R.A. 1973).

Table 1.3 A survey of attempts at immunological restoration by implantation of diffusion chambers and envelopes containing thymus tissue

Authors	Host.	Method of immune deprivation	Container, porosity	Origin of implanted thymus	Criteria for recovery.
Levey, R.H. et al 1963 a	C3Hf mice	NTx[1]	chamber, 0.45μ	newborn, syngeneic	prevention of lymphopenia and wasting
Osoba, D. and Miller, J.F.A.P. 1963	(CBA x T6)F_1 mice	NTx	envelope, 0.30μ	newborn or embryonic CBA	restoration of skin homograft rejection machanism
Levey, R.H. et al 1963 b	Swiss Webster mice	NTx	chamber, 0.45μ	newborn syngeneic	restoration of lethal reaction to LCM_2 virus.
Osoba, D. and Miller, J.F.A.P. 1964	(CBA x T6)F_1 mice	NTx	envelope, 0.30μ	newborn or embryonic CBA	prevention of wasting, increased lymphocyte numbers, restored ability to reject allogeneic skin grafts and to respond to sheep erythrocytes.
Law, L.W. et al 1964 a	C3H and DBA mice	NTx	chamber, 0.45μ	young, syngeneic	increased haemolysin titre to sheep erythrocyte challenge.
Miller, JF.A.P. et al 1964 a	C57BL mice	adult Tx[3] plus irradiation	chamber, 0.30μ	newborn, syngeneic	restored ability to reject C3H mouse skin homografts.
Barclay, T.J. 1964	(BALB/C x C57BL) F_1 mice.	adult Tx plus irradiation	chamber, 0.45μ and 0.10μ	newborn and adult syngeneic	restored skin allograft rejection mechanism.
Osoba, D. 1965 a	CBA mice	NTx	envelope, 0.10μ	syngeneic and allogeneic	prevention of wasting, increased antibody response to sheep erythrocytes and increased ability to reject skin allografts.

Table 1.3 continued

Authors	Host	Method of immune deprivation	Container porosity	Origin of implanted thymus.	Criteria for recovery
Schaller, R.T. and Stevenson J.K. 1967	C3H/HeJ mice	NTx	chamber, 0.30μ	multiple newborn syngeneic and allogeneic	recovery from wasting, restored skin allograft rejection mechanism and increased graft-versus-host reactivity of spleen cells
Osoba, D. 1968	CBA mice	adult Tx plus irradiation	envelope 0.10μ	newborn, syngeneic	increased spleen PFC^5 response to sheep erythrocytes.
Hallenbeck, G.A. et al 1969	C57BL/6 mice	NTx	chamber, 0.10μ	syngeneic	rejection of tumour xenograft (Walker 256)
Stutman, O. et al 1969 b	C3Hf/Bi mice	NTx	chamber, 0.22μ and 0.10μ	syngeneic or non-lymphoid thymoma	restored skin allograft rejection mechanism, restored delayed hypersensitivity to sheep erythrocytes, restored graft-versus-host reactivity of spleen cells
MacGillivray, M.H. et al 1964	Albino rats, random bred	NTx	unspecified	young rat	increased lymphocyte numbers in tissues, restored ability to reject skin allografts and to produce delayed hypersensitivity reactions to BSA_4

Table 1.3 continued

Authors	Host	Method of immune deprivation	Container porosity	Origin of implanted thymus	Criteria for recovery
Aisenberg, A.C. et al 1965	Sprague Dawley rats.	NTx	chamber, 0.45μ	young Sprague Dawley rat	prevention of wasting, restored ability to reject skin allografts and to produce delayed hypersensitivity reactions to BSA
Wong, F.M. et al 1966 and Sherman, J.D. 1967	Golden hamster	Tx at age of 2 weeks	envelope, 0.10μ	golden hamster	restored antibody response to human gamma globulin.
Trench, C. et al 1966	Rabbit	NTx	chamber, 0.45μ	autograft	restored blood lymphocyte count and anti-human gamma globulin titre
Biggart, J.D. 1966 a.b.	Wistar rats inbred	NTx	chamber, 0.45μ	neonatal, syngeneic	restored haemagglutination titre to sheep erythrocytes

Abbreviations:

1. NTx neonatal thymectomy
2. LCM lymphochoriomeningitis
3. Tx thymectomy
4. BSA bovine serum albumin
5. PFC plaque-forming-cell

IV. The pregnancy experiments

The idea behind these experiments originated from results showing that the immunological response of neonatally thymectomized mice was restored by intra-peritoneal implants of millipore diffusion chambers containing 14-day-old embryonic thymuses (Osoba, D. and Miller, J.F.A.P. 1963) (Osoba, D. and Miller, J.F.A.P. 1964). Osoba reasoned that if an embryonic thymus in a diffusion chamber was restorative then perhaps the foetal thymus in utero might similarly restore immune competence. (Osoba, D. 1973).

Osoba allowed 8 week-old female CBA mice, which had been thymectomized within a few hours after birth, to mate with normal T6 male mice. Another group of neonatally thymectomized female CBA mice were not allowed to mate and served as a control group along with a group of sham-thymectomized females. Of the 17 mated females, 9 became pregnant and delivered one or two litters prior to 13 weeks of age. The remaining 8 mice died of wasting disease before becoming pregnant or delivering any litters. When the mice were 13 to 17 weeks old they were challenged with sheep erythrocytes and given skin grafts from male mice of the T6 and Ak strains. It was found that 8 out of 9 parous females produced haemagglutination titres of a similar magnitude to those found in sham-operated control mice and that all the parous females rejected both Ak and T6 skin grafts within 15 days of grafting. The mean survival time of these grafts was the same as in sham-thymectomized mice. In contrast 11 of the 12 unmated, neonatally thymectomized mice retained their grafts for more than 15 days and 7 of them retained the grafts for more than 40 days. The immunological restoration of parous neonatally thymectomized mice was not associated with any significant change in

the total number of peripheral blood lymphocytes (Osoba, D. 1965 b).

Three possible mechanisms to account for the immunological restoration following pregnancy have been discussed (Osoba, D. 1973). The first was that foetal lymphoid cells crossed the placenta and seeded the maternal tissues where they were capable of reacting immunologically. This possibility was thought unlikely in view of the fact that the parous females rejected T6 skin grafts as readily as those from an unrelated strain. (If foetal cells, of genetic type (CBA x T6)F_1, had crossed the placenta they would not have been able to react against parental, T6 skin). Also, no T6 chromosomes were found, by cytological analysis, in the lymphoid tissues of six restored, parous CBA mice. The second possibility was that the hormonal changes accompanying pregnancy might be restorative. This was shown to be unlikely for the following reasons. A group of neonatally thymectomized female CBA mice were made pseudo-pregnant by the insertion of an applicator stick into the vagina. During pseudopregnancy corpora lutea develop in both ovaries and persist for 14 to 16 days producing some of the hormones associated with pregnancy. These mice behaved as unmated mice in that they retained skin grafts for longer than 15 days and produced low haemagglutination titres to sheep erythrocytes. In order to investigate the effect of placental hormones, Osoba mated neonatally thymectomized female CBA mice with T6 males. At 10, 14 and 18 days following the onset of pregnancy the female mice were anaesthetized and the foetuses removed via multiple hysterotomies. In half of this group the placenta was left in the uterus and in the other half the products of conception were removed in toto. Two weeks following the hysterotomies the mice were challenged with a T6 skin graft. All of the 8 mice undergoing hysterotomy at 10 days of gestation retained

the skin grafts for more than 15 days. Of the 9 mice which had undergone hysterotomy at 14 days, 5 rejected the grafts prior to 15 days of grafting. On the other hand, of the 14 animals which had undergone hysterotomies at 18 days of gestation 12 rejected the skin grafts within 15 days of grafting. This occurred regardless of whether the products of conception had been completely removed at hysterotomy or not. These experiments indicated that placental hormones do not have a restorative effect. Furthermore, restoration did not occur prior to 10 days gestation but had occurred by 18 days. The restoration therefore paralled the time period during which the foetal mouse thymus develops (Miller, J.F.A.P. and Davies, A.J.S. 1964). The third possibility was that the foetal thymus produced a humoral substance that crossed the placenta and brought about maturation of the maternal immunological system.

Since the original report by Osoba in 1965 (Osoba, D. 1965 b) nine years passed before confirmation of the restorative effect of pregnancy was obtained, this time in neonatally thymectomized rats (Borum, K. 1974). During the intervening period only one negative report had been published (Stutman, O. and Good, R.A. 1973) although perhaps several pregnancy experiments had been attempted.

Stutman and Good looked for immune restoration in neonatally thymectomized C3Hf and A female mice after three consecutive pregnancies following matings with A and C3Hf male mice, respectively (Stutman, O. and Good, R.A. 1973). At 17 weeks of age the surviving females were killed and their spleen cells used in graft-versus-host assays using various F_1 hybrid combinations as recipients. No restoration of graft-versus-host reactivity was found in any of the strain combinations tested. They did find, however, that pregnancy prolonged the survival

of neonatally thymectomized mice. This increased survival has been observed by others (Osoba, D. 1965 b) (Elders, M.J. et al 1968) (Borum, K. 1974).

Borum found that neonatally thymectomized Wistar/Fu rats produced consistently low primary haemolysin responses to sheep erythrocytes (Borum, K. 1972). When female, neonatally thymectomized, Wistar/Fu rats were mated with their brothers and challenged with sheep erythrocytes 15 - 55 days or 4 - 5 months after a single pregnancy a substantially restored antibody response was found (Borum, K. 1974). Unmated thymectomized and sham-thymectomized females and sham-thymectomized uniparous rats were included in the study as controls. This experiment showed that the beneficial immunological effect of a single pregnancy lasted at least 5 months. Blood lymphocyte levels were not published and no other criteria for immunological restoration apart from the response to sheep erythrocytes was used.

An observation which perhaps relates to the question of a functioning foetal thymus was made by Elders in 1968 (Elders, M.J. et al 1968). He compared the body and organ weights of newborn mice born from thymectomized and sham-thymectomized mothers and found no significant differences except for the thymus which was significantly larger in the offspring of thymectomized mothers. This might therefore indicate a homeostatic inter-relationship concerning thymic function between mother and foetus.

Further evidence for the transplacental passage of a thymic factor has been provided by Bach and co-workers who found that serum thymic activity reappeared in the blood of thymectomized mice during pregnancy (Bach, J.-F. et al 1975 a). In these mice no activity was detected until the 14th or 15th day of gestation when a progressive

increase in the level of thymic factor occurred, with a peak immediately before parturition. In the hours following birth the thymic factor level became nil again. However, the possibility has not been excluded that the "thymic factor activity" in the mother's serum might have been due to various secretions related to pregnancy including placental secretion.

Recently, the effect of pregnancy upon the immunological competence of congenitally athymic (nude) mice has been studied (Humber, D.P. et al 1975) (Jutila, J.W. et al 1975) (Pierpaoli, W. and Besedovsky, H.O. 1975). Female nude mice when mated with normal male mice produce litters of normal mice. Such a pregnancy in nude mice, in comparison to a pregnancy through a mating with nude male mice, in which the offspring are also thymusless, restored neither the PHA response of spleen cells nor the plaque-forming-cell response to sheep erythrocytes (Humber, D.P. et al 1975). Similar results have been found by other workers. (Jutila, J.W. et al 1975) (Pierpaoli, W. and Besedovsky, H.O. 1975). The implantation of a thymus within a millipore diffusion chamber was found not to restore the immunological competence of nude mice (Jutila, J.W. et al 1975) (Pierpaoli, W. and Besedovsky, H.O. 1975). Perhaps the reason for the failure to restore the nude mouse lies in the fact that, unlike the neonatally thymectomized mouse, the nude mouse has not had the benefit of thymic action in foetal and neonatal life and does not contain any cells "processed" by the thymus. Another possibility is that the immunological deficiency in nude mice is not entirely attributable to thymic aplasia. Also, the reverse of the process postulated by Osoba apparently does not occur in the nude mouse. Nude mice are usually bred from nu/+ mothers which have normal thymus glands

and yet no restoration of immunological competence occurs in the offspring (Humber, D.P. et al 1975). This failure of the nude foetus to benefit from its mother's thymus could be caused by the failure of the humoral factor to cross the placenta from mother to foetus or through the inability of the foetus to respond.

In summary, the mechanism by which pregnancy influences immune competence is still obscure. The transplacental passage of a humoral substance from the foetal thymus gland remains a plausible explanation for maternal immune restoration.

V. Thymic extracts.

The aim of this section is to review the work performed with thymic extracts after 1949 (the historical section of this chapter contains a review of thymic extracts before 1949). Recent literature on the thymus and thymic extracts has been vast and many review articles have been published (Metcalf, D. 1966) (Miller, J.F.A.P. and Osoba, D. 1967) (White, A. and Goldstein, A.L. 1968) (Davies, A.J.S. 1969 a) (Luckey, T.D. 1973) (Trainin, N. 1974) (Friedman, H. 1975) (Van Bekkum, D.W. 1975).

Some of the biological effects of various thymic extracts are listed in table 1.4. Thymic extracts have been reported to produce hypoglycaemia (Pansky, B. et al 1965), to affect calcium and phosphate metabolism (Schwarz, H. et al 1953) (Potop, I. et al 1966) (Milcu, S.M. and Potop, I. 1973) (Mizutani, A. 1973), to affect muscle contraction and myasthenia (Constant, G.A. et al 1949) (Wilson, A. and Wilson, H. 1955) (Parkes, J.D. and McKinna, J.A. 1967) (Goldstein, G. and Manganaro, A. 1971) and to have various growth inhibitory properties (Dubos, R.J. and Hirsch, J.G. 1954) (Szent-Gyorgyi, A. et al 1962)

Table 1.4 A survey of thymic extracts and their biological effects from 1949 to 1976.

Year	Authors	Type of extract	Biological effect
1949	Roberts, S. and White, A. 1949.	calf thymus, aqueous extract, ethanol-insoluble fraction.	stimulation of lymphopoiesis and thymic hyperplasia in adult rats.
1949	Constant, G.A. et al 1949.	human, dog and cat thymus, saline extract.	reduced cat muscle contraction in vivo and in vitro.
1951	Loefer, J.B. and Gilles, N.G. 1951.	foetal bovine thymus, saline extract.	greater number of tumour takes in extract-treated rats.
1953	Eskelund, V. and Plum, C.M. 1953.	calf thymus extracted with ethanol and then with ether.	accelerated healing of bone fractures (other tissue extracts also effective).
1953	Schwarz, H. et al 1953.	calf thymus extracted with ether then water and lyophilized.	increased serum calcium and phosphate levels in man and dog.
1954	Dubos, R.J. and Hirsch, J.G. 1954.	calf thymus, acid extract.	inhibition of growth of mammalian mycobacteria.
1955	Wilson, A. and Wilson, H. 1955.	calf, human and foetal whale thymus, acetone and saline extraction, lyophilized.	depression of muscle contraction in isolated rat phrenic nerve-diaphragm, temporary rigidity in chicks and mice following iv administration.
1956	Gregoire, Ch and Duchateau, Gh 1956	irradiated pig and rabbit thymus, saline extract.	lymphoid hyperplasia in regional lymph nodes in rat.
1956	Metcalf, D. 1956.	mouse and human thymus saline extract (LSF)	blood lymphocytosis in young mice.
1957	Nakamoto, A. 1957 a b.	bovine thymus, lipid-rich fraction.	blood lymphocytosis in young rabbits.
1962	Duplan, J.F. et al 1962.	mouse thymus, saline extract.	increased lymphocyte to poly-morphonuclear leukocyte ratio.
1962	Szent-Gyorgyi, A. et al 1962.	calf thymus, multiple extractions, two	promotion or retardation of tumour

Table 1.4 continued

Year	Authors	Type of extract	Biological effect
1962	Szent-Gyorgyi, A. et al 1962.	fractions 'promine' and 'retine'.	growth, induction of temporary sterility.
1964	Camblin, J.G. and Bridges, J.B. 1964.	rat and rabbit thymus, homogenised in saline.	restoration of lymphocyte level in blood of young, X-irradiated rats.
1964	Maisin, J.H.F. 1964.	neonatal mouse thymus saline extract.	protection of mice against the carcinogenic action of methylcholanthrene.
1964	Schooley, J.C. and Kelly, L.S. 1964	rat thymus, saline extract.	increased weight of lymph nodes in NTx rats.
1965	Pansky, B. et al 1965.	mouse thymus, acetone extract.	marked hypoglycaemic effect in young mice.
1965	Jankovic, B.D. et al 1965 a.	rat thymus extracted with saline then methanol and chloroform.	reduced incidence of wasting, blood lymphocytosis, improved delayed response to BSA in NTx rats.
1965	Klein, J.J. et al 1965.	mouse, rat and calf thymus, saline extract (Thymosin).	increased weight of lymph node and spleen, enhancement of uptake of tritiated thymidine into DNA of lymph nodes.
1966	Trainin, N. et al 1966.	sheep, calf, and rabbit thymus, saline extract (Thymus humoral factor).	stimulation of lymphopoiesis and prevention of wasting in NTx rats.
1966	Potop, I. et al 1966.	calf thymus, lipid and protein fractions.	increased ATP content, decreased inorganic phosphate content in muscle and serum of thymectomized rabbits.
1967	Hand, T. et al 1967.	calf thymus, saline extraction, ammonium sulphate fractionation. (lymphocyte stimulating hormone).	blood lymphocytosis in young mice.

Table 1.4 continued.

Year	Authors	Type of extract	Biological effect
1967	Brunkhorst, W. and Herranen, A. 1967.	rat thymus, extract of thymocyte fraction and reticular fraction	injection of thymocyte extract led to delayed rejection of skin grafts in NTx rats.
1967	Parkes, J.D. and McKinna, J.A. 1967	human myasthenic thymuses extracted with acetone or saline.	neuromuscular block in rat sciatic nerve preparation.
1968	Law, L.W. et al 1968.	mouse thymus, saline extract.	restoration of graft-versus-host activity of spleen cells from NTx mice.
1971	Carpenter, C.B. et al 1971 a.	bovine thymus, homogenised in saline, fractionation by DEAE chromatography.	redueed isotope uptake in lymphocytes responding to PHA. Reduced haemagglutinin response in mice injected with sheep erythrocytes.
1971	Goldstein, G. and Manganaro, A. 1971.	bovine thymus, saline extract, peptides purified by chromatography.	neuromuscular block following injection into rats.
1971	Milcu, S.M. and Potop, I. 1971.	calf, horse and human thymus; peptide and lipid fractions.	antimitotic effect in vitro and in vivo.
1971	Kiger, N. 1971.	calf thymus, aqueous extract.	inhibition of DNA synthesis in lymphoid cell culture, decreased PFC response following injection of sheep erythrocytes.
1971	Houck, J.C. et al 1971.	calf and rat thymus, sonicated in saline, centrifuged, dialysed and lyophilized.	inhibition of in vitro response to PHA (Spleen and other lymphoid extracts also active).
1972	Sudo, T. et al 1972.	calf thymus extracted with sulphuric acid.	stimulation of lymphopoiesis and reduced deaths from wasting in NTx mice.

Table 1.4 continued

Year	Authors	Type of extract	Biological effect
1972	Robey, G. et al 1972.	bovine thymus, saline extract, precipitation with ammonium sulphate and methanol.	acceleration of appearance of haemolysin to sheep erythrocytes in young mice.
1973	Wilson, O.H. and Bhaumick, B. 1973.	calf thymus, ammonium sulphate precipitation, DEAE chromatography and gel filtration.	restoration of ability to reject skin grafts in Tx mice, increased isotope uptake in mouse spleen, induction of θ antigen in vitro.
1973	Comsa, J. 1973.	veal thymus, glycopeptide (Homeostatic thymus hormone).	prevention of consequences of thymectomy in guinea-pigs and rats.
1973	Milcu, S.M. and Potop, I. 1973.	calf thymus, acetone extract. (Thymus polypeptide).	stimulation of calcium and phosphate deposition in bone, stimulation of metabolism, in vivo anti-tumour effect, stimulation of lymphopoiesis and erythropoiesis.
1973	Mizutani, A. 1973.	bovine thymus, saline extract, ammonium sulphate fractionation	hypocalcaemic effect in rabbit, some lymphopoietic activity.
1975	Olsson, L. and Claesson, M.H. 1975	mouse thymus, frozen tissue extracted in water and lyophilized.	reduced mitotic activity of mouse spleen (spleen extract similar but weaker effect).

Abbreviations:

NTx Neonatally thymectomized.
Tx thymectomized.
PFC plaque-forming cell.

(Kiger, N. 1971) (Houck, J.C. et al 1971). In this thesis, however, only those thymic extracts having an effect on the structure or function of the lymphoid tissues are discussed in any depth.

(A) Effect on lymphoid tissue structure.

In 1949 Roberts and White demonstrated a lymphocytopoietic factor in fractions of calf, rabbit and rat thymus prepared by the cold ethanol technique of Cohn and co-workers. A saline-soluble fraction obtained from calf thymus, rich in nucleoprotein, produced a marked lymphocytosis and increased the mass of lymphoid tissue in adult rats (Roberts, S. and White, A. 1949). Gregoire and Duchateau found that a cell-free saline extract prepared from X-irradiated, and therefore largely lymphocyte-depleted, pig or rabbit thymus, produced a lymphoid hyperplasia in adult rats (Gregoire, Ch. and Duchateau, Gh. 1956). Control extracts prepared from lymph nodes or muscle were inactive. Metcalf prepared extracts of various human and mouse tissues, by grinding the tissues with silica at room temperature, suspending the mixture in saline and removing the cell debris by low speed centrifugation (Metcalf, D. 1956 c). Mouse or human thymic extracts produced a blood lymphocytosis in young mice following intracerebral injection (Metcalf, D. 1956 c) or following subcutaneous injection into adult thymectomized mice (Metcalf, D. 1959). Injection of extracts of mouse lymph node, spleen, liver, kidney, lung, muscle and brain did not produce a lymphocytosis (Metcalf, D. 1956 c). Metcalf thought that the most probable source of the "lymphocyte stimulating factor (LSF)" was the epithelial cells of the thymus (Metcalf, D. 1956 c) (Metcalf, D. 1958). The LSF was found to be non-dialysable and heat-labile since heating at 60°C. destroyed its activity. In 1957 Nakamoto reported that a lipid-rich extract of boving thymus produced

a blood lymphocytosis when injected into young rabbits (Nakamoto, A. 1957 a c). Other extracts tested were inactive. Duplan and co-workers in an attempt to confirm Metcalf's findings, prepared mouse LSF according to Metcalf's procedure and injected it intracerebrally into newborn inbred mice. An initial success in increasing the lymphocyte to polymorphonuclear leukocyte ratio in the blood was reported, but later following some change in the test mice, Duplan was unable to reproduce his earlier results (Duplan, J.F. et al 1962). Metcalf's preparative method was also used by Schooley who prepared a rat thymus extract which was found to increase the lymph node weight in neonatally thymectomized rats (Schooley, J.C. and Kelly, L.S. 1964). This effect was not specific to the thymus as lymph node extracts were also active.

In 1964 Camblin and Bridges reported a lymphocytosis following the injection of a rat or rabbit thymic extract into young rats which were lymphopenic after having received a small dose of total body irradiation and a large dose of thymic irradiation (Camblin, J.G. and Bridges, J.B. 1964). Heated thymic extracts and extracts of spleen and brain were not effective in restoring white cell counts. Jankovic and co-workers tested a lipid-rich extract of rat thymus by injection into inbred neonatally thymectomized rats and observed a blood lymphocytosis (Jankovic, B.D. et al 1965 a). A control lipid fraction prepared from nervous tissue was without effect. Thymic extract injections prevented wasting in the neonatally thymectomized rats but did not improve the cellular depletion in the spleen and other lymphoid organs.

Klein, Goldstein and White found that cell-free thymic extracts prepared from calf, rat and isologous mouse tissue stimulated lymphopoiesis, measured by the uptake of radioactive precursors by the

lymphoid organs of normal (Klein, J.J. et al 1965) (Klein, J.J. et al 1966) and X-irradiated mice (Goldstein, A.L. et al 1970 c). After repeated injections of the thymic extract, increased lymph node and spleen weights were also noted. Bovine serum albumin (BSA) and extracts of rat lymph nodes and spleen did not produce similar effects (Klein, J.J. et al 1966). Administration of a more pure thymic extract preparation, thymosin fraction 3, to neonatally thymectomized mice three times a week for nine weeks increased the number of lymphoid cells in the peripheral blood, lymph nodes and spleen. In comparison, neonatally thymectomized mice treated with saline, BSA or other calf extracts such as spleen or liver, or lipopolysaccharide antigens such as the endotoxin from Salmonella enteritidis did not show increased numbers of lymphocytes (Asanuma, Y. et al 1970) (Goldstein, A.L. and White, A. 1971). Also animals treated daily for seven days with thymosin prior to X-irradiation, initially had larger than normal lymphoid structures, which were less severely involuted following irradiation and subsequently showed an accelerated rate of regeneration compared to those of untreated, irradiated controls (Goldstein, A.L. et al 1970 c). Thymosin was also found to increase the number of lymphocytes in adrenalectomized and germfree mice (Goldstein, A.L. and White, A. 1970).

A cell-free extract prepared from calf, sheep or rabbit thymus by Trainin and co-workers was found to produce a blood lymphocytosis when injected into neonatally thymectomized, adult thymectomized and normal mice. Control extracts of sheep liver, rabbit kidney and calf muscle were ineffective. Enlargement of splenic lymphoid follicles, hyperplasia and an increased number of mitotic figures in the lymphoid tissue was observed in intact mice. Hyperplasia and increased mitosis

in the lymphocyte-depleted areas of the spleen of thymectomized mice was also observed following thymic extract treatment (Trainin, N. et al 1966). Incorporation of tritiated thymidine into the DNA of lymph node cells was increased following three daily thymic extract injections (Trainin, N. et al 1967 a). In this last assay calf kidney extract was also active.

A basic protein extracted from bovine thymus was called "lymphocyte stimulating hormone (LSH)" after the finding that an injection of LSH into young mice led to an increased number of blood lymphocytes. The increase in lymphocyte number was assessed by measuring the ratio between lymphocytes and polymorphonuclear leukocytes in the blood (Hand, T. et al 1967).

In summary, a large number of thymic extracts have been reported to have a stimulating effect on the number of circulating lymphocytes and the lymphoid tissue mass. Assays based upon changes in lymphocyte numbers, both in the blood and tissues, have to be vigorously controlled for the following reasons. Blood lymphocyte levels of small laboratory animals are known to fluctuate from one period of the year to another and moreover, apparently trivial procedures, such as puncturing the skin at injection, can drastically alter the blood lymphocyte level (Metcalf, D. 1966). As heterologous thymic extracts were used in most of the above experiments the effect of the administration of foreign protein remains an unknown factor in these experiments. Large amounts of heterologous protein might well have induced a non-specific stimulation of the lymphoid tissue.

(B) Effect on immune response

Following the clarification of the role of the thymus by the neonatal thymectomy experiments of Miller and Good (Miller, J.F.A.P.

1961) (Good, R.A. et al 1962) and the realisation that the morphological changes seen in the lymphoid system following the injection of heterologous thymic extracts were difficult to interpret, the emphasis shifted from the evaluation of thymic preparations by morphological criteria to assays of functional parameters.

The post-thymectomy wasting syndrome, thought to be caused by the immunological incompetence of the host has been prevented in neonatally thymectomized mice (Trainin, N. et al 1966) (Asanuma, Y. et al 1970) (Goldstein, A. et al 1971) (Sudo, T. et al 1972), rats (Jankovic, B.D. et al 1965 a) and guinea-pigs (Comsa, J. 1973) by injections of cell-free thymic extract. Not all thymic extracts, however, prevented the occurrence of wasting (Law, L.W. and Agnew, H.D. 1968).

One of the first experiments suggesting that immunological repair had occurred in thymus-deprived animals injected with a cell-free thymic extract was that performed by Jankovic and co-workers (Jankovic, B.D. et al 1965 a). Neonatally thymectomized rats were treated with injections of either a thymic extract or an extract of central nervous tissue. The rats were then immunised with BSA in complete Freunds adjuvant and skin tested with BSA 10 and 18 days later. The response of the thymic extract-treated rats had improved at the time of the first test and was restored to normal by the second test. Rats injected with the control extract had only slightly improved responses.

When "thymic humoral factor" (THF) was injected into newborn thymectomized or adult thymectomized, irradiated mice the ability to reject skin grafts was restored (Trainin, N. and Linker-Israeli, M. 1967). Repeated injections of THF into adult thymectomized and

irradiated mice allowed the survival of the mice following the transplantation of an allogeneic fibrosarcoma that killed the untreated thymectomized controls. This immunological repair was not observed in thymus-deprived mice injected with calf muscle or kidney extracts (Trainin, N. and Linker-Israeli, M. 1967). Large doses of thymosin administered to normal B10D2/Sn mice shortened the first- and second-set allogeneic skin graft rejection times (Hardy, M.A. et al 1968). Injections of thymosin fraction 3 restored the ability of neonatally thymectomized mice to reject allogeneic skin grafts. Neonatally thymectomized mice injected with saline, BSA or a spleen extract retained their grafts over the experimental observation period (Goldstein, A.L. et al 1970 a) (Goldstein, A.L. et al 1970 b). More recently, Wilson and Bhaumick reported that injections of an ammonium sulphate precipitate of a calf thymic extract permitted thymectomized, irradiated, bone marrow protected mice to reject allogeneic skin grafts in the same time as normal rats (Wilson, O.H. and Bhaumick, M. 1973).

The potential of spleen cells to induce a graft-versus-host reaction when injected into suitable recipients is known to be reduced by neonatal thymectomy (Dalmasso, A.P. et al 1962 a). Repeated injections of THF partially restored the capacity of lymphoid cells from neonatally thymectomized mice to induce in vivo splenomegaly (Trainin, N. and Linker-Israeli, M. 1967) (Trainin, N. et al 1967 b) and runting disease (Trainin, N. et al 1967). Law and Agnew repeatedly injected a cell-free mouse thymus homogenate into syngeneic newborn thymectomized mice before testing the competence of the spleen cells to induce runting in newborn BALB/c mice (Law, L.W. and Agnew, H.D. 1968). Although injections of this thymic extract neither

reduced the incidence of wasting nor elevated blood lymphocyte counts, the lymphoid cells of these mice were more capable of producing graft-versus-host reactions than cells from untreated control mice. Law also tested a batch of thymosin fraction 3, prepared by Goldstein and White, and found that repeated injections into neonatally thymectomized C57BL/KaLw mice permitted the development of spleen cells capable of performing graft-versus-host reactions, measured by runting and death of the recipients when the treated animals were sacrificed at five weeks of age. A similarly prepared spleen extract was inactive and, as before (Law, L.W. and Agnew, H.D. 1968) thymosin treatment did not affect wasting or the peripheral blood lymphocyte count in neonatally thymectomized mice (Law, L.W. et al 1968).

Small and Trainin found that repeated injections of a calf thymus extract into neonatally thymectomized C3H/eb mice led to a significantly greater number of haemolysin plaque-forming cells (PFC's), responding to sheep erythrocytes, in the spleens of treated mice. A similarly prepared calf kidney extract was inactive (Small, M. and Trainin, N. 1967). In contrast, repeated injections of thymosin into neonatally thymectomized mice was found to raise only slightly the number of antibody-forming cells in the spleens of mice injected with sheep erythrocytes, This increase in the number of PFC's was not considered to be statistically significant (Goldstein, A.L. et al 1970 a). These thymosin-treated mice were, however, capable of rejecting allogeneic skin grafts (Goldstein, A.L. et al 1970 a). Also no significant change was found in the level of circulating antibody to sheep erythrocytes in normal mice or in X-irradiated mice pre-treated with thymosin before antigen challenge or in those mice given thymosin during the period of immunization (White, A. and Goldstein, A.L. 1970).

There is considerable evidence that the control of neoplasia is related to host immunological competence. Maisin found that mice either repeatedly grafted with neonatal thymuses or injected with a crude thymus homogenate subsequently had a lower incidence of skin cancer compared to untreated mice following painting of the skin with 20-methylcholanthrene (Maisin, J.H.F. 1964). Maisin attributed the lower incidence of skin cancer to an increase in immunological resistance of the treated mice. Szent-Gyorgyi and co-workers obtained two active fractions, "retine and promine", by ethanol-chloroform extraction of calf thymus glands (Szent-Gyorgyi, A. et al 1962). Retine was found to inhibit tumour growth whilst promine promoted tumour growth. It was later found, however, that these factors could be obtained from other tissues,,such as aorta, muscle and tendon and were therefore not specific to the thymus (Szent-Gyorgyi, A. et al 1963).

Full immunological competence is not attained in laboratory rodents until several weeks after birth and several studies have shown that the ability to perform certain immune reactions is accelerated, in young mice, by injections of thymic extracts. For example, Hand and co-workers found that injection of a crude and purified saline extract of calf thymus stimulated and accelerated the development of the capacity of newborn mice to respond to sheep erythrocytes (Hand, T. et al 1968) (Hand, T. et al 1970). Another purified fraction suppressed markedly the development of antibody-forming cells in neonatal mice and simultaneously inhibited lymphopoiesis. This inhibitory effect was only temporary since older mice injected at birth with the same fraction responded normally to sheep erythrocyte

challenge (Hand, T.L. et al 1970). Goldstein and co-workers found that administration of thymosin fraction 3 to newborn mice accelerated the development of the capacity of the cells to elicit a graft-versus-host response (Goldstein, A.L. et al 1971). Similarly a single injection of thymosin into newborn mice, 24 hours before killing, accelerated the onset of PHA responsiveness, measured in vitro by tritiated thymidine incorporation (Goldstein, A.L. and White, A. 1973). Additional evidence for the role of a thymic factor in the development of cell-mediated immunological competence was obtained in experiments using the Moloney sarcoma virus (MSV). The lethality of this virus is related to the state of the host's cell-mediated immune competence. For example, in newborn or immune-deprived mice the tumour will grow progressively and kill the host. In mice aged five weeks or more, however, no mice will succumb to the tumour. At some time between the second and third week of life, the host becomes competent to resist progressive tumour growth. It was found that calf thymosin given to normal mice from birth until two weeks of age significantly accelerated the development of the ability to resist tumour growth (Zisblatt, M. et al 1970). Extracts from calf spleen or liver did not affect the development of host resistance to the oncogenic MSV. Similarly, thymosin administration to adult mice, immuno-suppressed by anti-lymphocyte serum or by combined thymectomy and irradiation, restored in part the capacity to resist tumour growth (Hardy, M. et al 1971). As yet, relatively few in vivo experiments have been performed with the serum thymic factor (TF) of Jean-Francois Bach and co-workers. Injections of purified serum TF into thymectomized, irradiated, bone marrow cell reconstituted mice at the same time as an intramuscular injection of MSV allowed most of

the treated mice to resist tumour growth (Bach, J-F. et al 1975 c). The effect of the thymic factor treatment appeared to be reversible, however, as many mice had tumour recurrence after the treatment had stopped.

In none of the attempts to replace the influence of the thymus by a thymus-derived humoral factor, either by thymus contained in millipore diffusion chambers or by the administration of thymic extracts has full immunological restoration been observed. Several possible explanations exist for this lack of complete restoration. The dose and duration of the replacement therapy might not be optimal. The thymic factor preparation might need additional factors, or have lost co-factors, necessary for its full action. A local high concentration of the thymic factor in a specialised microenvironment might be necessary for its full action. Alternatively, it could be argued that the slight restoration of thymus-deprived animals by the administration of thymic extracts could be due to non-specific effects. Evidence in support of the latter theory comes from several published experiments in which no effect of a thymic factor was found. For example, no effect was demonstrated using crude saline extracts of mouse or rat thymus (Dalmasso, A.P. et al 1963) (Miller, J.F.A.P. 1964 b) (Jankovic, B.D. et al 1962). Attempts to demonstrate an effect of thymic extracts by Davies and co-workers was also unsuccessful (Davies, A.J.S. 1975). Negative fiudings have also been reported with thymosin, probably the best documented thymic factor (Kruger, J. et al 1970) (Lance, E.M. et al 1973). Moreover, in a critical review on thymic hormones Stutman and Good presented results showing that no immunological restoration was found in immune deprived mice following treatment with thymosin fraction 3 and various other thymic extract preparations

(Stutman, O. and Good, R.A. 1973). The thymosin fraction studied by Stutman and Good was provided by Allan Goldstein. These results included no restorative effect on the response to sheep erythrocytes in neonatally thymectomized or adult thymectomized, irradiated mice; no restoration in the capacity of spleen cells from neonatally thymectomized mice to perform graft-versus-host reactions and no restored ability to reject allogeneic skin grafts (Stutman, O. and Good, R.A. 1973).

The presence of endotoxin from bacterial contamination, a common contaminant of tissue preparations, might be an explanation, in some instances, for the restorative effect of thymic extracts. For example, endotoxin was found markedly to enhance the antibody response of normal and sublethally irradiated rabbits when given separately or in conjunction with protein antigens (Johnson, A.G. et al 1956) (Kind, P. and Johnson, A.G. 1959). Also the first-set skin homograft rejection time was shortened in normal rabbits (Al-Askari, S. et al 1964) and mice (Stutman, O. and Good, R.A. 1973) given endotoxin. In a study made by Kruger and colleagues, two batches of bovine thymosin were thought to be contaminated by endotoxin (Kruger, J. et al 1970). In this study thymosin treatment failed to restore the capability of thymectomized, irradiated rats to produce serum antibodies to bovine gamma globulin and also no restoration of the lymphoid-depleted tissues was observed. Thymosin, when given with complete Freund's adjuvant, was found to be highly immunogenic in rats, giving rise to delayed reactions and antibody formation. In view of the failure to restore immune reactivity with thymosin the authors thought that perhaps previous positive results obtained with thymic extracts might have been caused by their immunogenic, adjuvant or endotoxin-like properties.

The specificity of the effect of thymic factors in accelerating tumour rejection can also be questioned since the effects of various non-specific stimulators on the host response to tumours is well known (Yashphe, D.J. 1970). The tumour-growth inhibitory factor retine is an example of a non thymus-specific factor affecting tumour growth (Szent-Gyorgyi, A. et al 1963). Also, an acceleration in the development of immune reactivity in newborn mice by thymic factor treatment (Hand, T. et al 1968) (Zisblatt, M. et al 1970) (Goldstein, A.L. et al 1971) has been achieved to a comparable degree with endotoxin, synthetic polynucleotides and deoxyribonucleotides (Hechtel, M. et al 1965) (Winchurch, R. and Braun, W. 1969). Recent observations that the immune reactivity of thymectomized mice could be restored by the injection of synthetic polyanions and polynucleotides (Cone, R.E. and Johnson, A.G. 1971) (Diamantstein, T. et al 1971) further questions the significance of the restorative effects of thymic extracts.

In summary, there is no unanimity amongst the various investigators regarding the extent to which thymic humoral factors can replace normal thymic function. The reasons for such differences include the diverse chemical characteristics of the various extracts, the use of different experimental animals and the method and the extent of immune-deprivation in the test animal. Partly because of these difficulties investigators have also developed in vitro assays of thymic factor activity; some of these assays are discussed in a later section of this chapter.

(C) Physical and chemical characteristics of some thymic extract preparations.

A summary of the physical and chemical characteristics of some of the more extensively studied products obtained from the thymus

gland and from serum is shown in table 1.5. Considerable variation appears to exist between the chemical characteristics of the various thymic factor preparations. This lack of agreement may reflect the diverse methods of isolation or the existence of more than a single thymic factor, or both. It is possible that, at the present state of purification, the thymic factors may be in a state of aggregation or bound to other molecules, such that, on further purification the factors will resemble one another more closely. However, from comparing the amino acid composition of thymosin fraction 8 (Goldstein, A.L. et al 1975) and thymus humoral factor (THF) (Kook, A.I. et al 1975) it does not appear that thymosin is an aggregate of THF. Amino acid analysis of the serum thymic factor (TF) has not yet been accomplished. Thymosin fraction 5, the preparation used for most of the biological studies to date, has been shown to contain at least 11 heat-stable acidic peptides and proteins with molecular weights ranging from 1,200 to 14,000 (Goldstein, A.L. et al 1975 a). It is not known whether all of the biological activity ascribed to thymosin resides within a single molecular species or whether there exists a family of thymus-specific molecules acting together to endow the host with its normal immunological capacity. The possibility that several of the factors present in thymosin fraction 5 might be biologically active is suggested from observations showing that, in some in vitro systems, thymosin fraction 8 preparations are not as active as fraction 5 preparations (Hooper, J.A. et al 1975).

Previous experience with insulin has shown that the physical and chemical characteristics of the molecule synthesized in the endocrine gland are different from those of the secreted moiety. The same could be true for the thymic hormone(s). This would explain the difference between the serum thymic factor and the factors isolated from the thymus

Table 1.5 Physical and chemical properties of some thymic factors.

Name of factor	Chemical class	Molecular weight	Properties	Reference
Thymosin(fraction 8)	protein	12,200	108, amino acids, heat stable.	Hooper, J.A. et al 1975.
Thymus humoral factor (THF)	peptide	3,200	31 amino acids, high proportion of acidic residues, heat stable.	Kook, A.I. et al 1975.
Circulating thymic factor (TF)	peptide	1,000	unstable at room temperature pK_I 7.5	Bach, J-F. et al 1975 a.
Thymopoietin I) Thymopoietin II)	protein	7,000	heat stable, N-terminus: glycine, C-terminus: lysine.	Goldstein, G. 1975.
Homeostatic thymus hormone (HTH)	glyco-peptide	2,000	heat stable	Comsa, J. 1973.
Lymphocyte stimulating hormone (LSH_h)	protein	15,000	heat labile	Luckey, T.D. and Venugopal, B. 1975.
Lymphocyte stimulating hormone (LSH_r)	protein	80,000	heat stable	Robey, W.G. 1975.

gland. Further work on the purification and chemical analysis of the various thymic factors is necessary and eventually the many apparently different compounds may well fall into simple perspective.

(D) Thymic factors in serum.

A prerequisite for establishing an endocrine function for an organ is the demonstration that a secreted product of that organ circulates in the bloodstream. The presence of a thymus-specific factor (TF) in the serum of humans and animals has been demonstrated by means of a sheep erythrocyte rosette assay (Bach, J-F. et al 1975 c). In normal mice this factor disappears rapidly from the serum following thymectomy and is not present in the serum of athymic (nude) mice. Serum TF levels were restored to normal following thymus transplantation either as a free graft or enclosed within a cell-impermeable millipore diffusion chamber (Bach, J-F. et al 1973) (Dardenne, M. et al 1974). Furthermore, following a single injection of thymosin into thymectomized mice the level of serum TF became normal within a few hours and then fell (Hooper, J.A. et al 1975).

The development of a radioimmunoassay for bovine (Schulof, R.S. 1972) and human (Schulof, R.S. et al 1973) thymosin allowed further confirmation of the presence of thymosin in the blood of several mammalian species.

The demonstration of a thymic factor in the blood strengthens the hypothesis that the thymus functions not only by influencing the maturation of immature lymphoid cells within the thymus but also via a humoral factor maintaining or affecting the state of maturation of peripheral lymphoid cells.

VI Thymus perfusion experiments.

Several groups have developed procedures in which the isolated thymus of a small laboratory animal can be maintained, in vitro, for several hours, during which time the gland is perfused with tissue culture medium or diluted blood via the arterial system (Folkman, J. et al 1968) (Burleson, R. and Levy, R.H. 1971) (Ekueme, O. 1973) (Wallace, I.W.J. and Ekueme, O. 1972). The experimental results obtained by the above workers regarding the mechanism of action of the thymus are not in agreement. The lack of agreement undoubtedly reflects the use of different perfusion systems, different experimental animals and the different immunological tests used.

Levy and Burleson reported that the passage of immunoincompetent mouse lymphoid cells through either a syngeneic or a rat thymus restored the immunocompetence of the cells with respect to graft-versus-host performing ability and ability to break transplantation tolerance (Levy, R.H. and Burleson, R. 1975). Incubation of the immature mouse lymphoid cells in a perfusate, which had previously percolated through the gland for up to 48 hours, failed to confer this reactivity. Ekueme found that injection of the cell free medium obtained after perfusion through a rabbit thymus into neonatally thymectomized rabbits, restored the capacity to produce a delayed hypersensitivity response to BSA. Injection of thymus perfusate, however, had no restorative effect on the low peripheral blood lymphocyte count (Ekueme, O. and Forrest, A.P.M. 1974). Wallace injected the cell-free medium obtained from the perfusion of rat thymuses into syngeneic neonatally thymectomized rats and observed an increased blood lymphocyte count (Wallace, I.W.J. and Ekueme, O. 1972) and a restored ability to reject skin allografts (Wallace, I.W.J. personal communication).

The results of Levy and Burleson (Levy, R.H. and Burleson, R. 1975)

suggest that physical traffic through the thymus gland is the necessary event for the development of immunocompetence. The results of Ekueme and Wallace, however, suggest that a thymic humoral factor may be effective in increasing the immunological competence of thymus-deprived rodents (Wallace, I.W.J. and Ekueme, O. 1972) (Ekueme, O. and Forrest, A.P.M. 1974).

T CELL DIFFERENTIATION AND IN VITRO ASSAYS FOR THYMIC FACTORS.

This section will review T cell differentiation with particular regard to possible regulation by thymic humoral factors. Most of the published experimental work has been done with mouse and human tissue and therefore although the details may not be directly applicable to the rat it would be surprising if the generalities did not apply.

The thymus is believed to exert its immune function via the production of immunocompetent T lymphocytes. Thymus lymphocytes develop from stem cells that have migrated to the thymus from the yolk sac and liver in embryonic life (Moore, M.A.S. and Owen, J.J.T. 1967) (Owen, J.J.T. and Ritter, M.A. 1969) and from the bone marrow in adult life (Ford, C.E. et al 1966). Within the thymus lymphopoiesis is extremely active leading to the production of small, non-dividing lymphocytes with a calculated intra-thymic lifespan of not more than three or four days (Metcalf, D. 1966). The intense proliferative activity of thymic lymphocytes implies the operation of a potent stimulus thought to emanate from the thymus epithelium. This has been suggested by histological studies in mice (Metcalf, D. 1966) and guinea-pigs (Mandel, T. 1969) in which epithelial cells were found to be associated with statistically greater numbers of mitotic lymphoid cells than due to chance associations. Lymphopoiesis, within the

thymus, appears to exceed the export of thymocytes and this has led to the suggestion that most of the cells "born" in the thymus die there (Metcalf, D. 1966) (Matsuyama, M. et al 1966). However, especially as there is no evidence of massive cell death within the thymus, these views are not universally held (Sainte-Marie, G. 1973). It has been shown, however, that some cells, at least, leave the thymus to seed the peripheral lymphoid tissue (Murray, R.G. and Woods, P.A. 1964) (Nossal, G.J.V. 1964) (Weissman, I.L. 1967) (Davies, A.J.S. 1969 b).

It is possible to divide the mouse thymocyte population into subpopulations based on a number of physical, biological and immunological criteria. One such division is into large and medium sized dividing lymphocytes, which constitute about 10% of the population, and into small, non-dividing lymphocytes, that are thought to be the product of the former, and represent the majority of the thymocytes (Metcalf, D. 1966). Another division is based upon cell surface antigens. A major population, about 85%, has an antigenic constitution unique to the thymus in possessing, in the appropriate mouse strain, the TL antigen. These cells also have high levels of Thy-1 (θ) and low levels of H-2. The remaining, minor population has antigenic properties similar to peripheral T cells, namely absence of TL, relatively low levels of Thy-1 (θ) and high levels of H-2 (Cerottini, J-C. 1967) (Raff, M.C. and Cantor, H. 1971) (Schlesinger, M. 1972) (Cantor, H. and Weissman, I. 1976). The major, TL-positive population is believed to be primarily cortical thymocytes and to correspond to the cortisone-sensitive cells (Those cells which disappear from the thymus after an in vivo injection of cortisone acetate) (Blomgren, H. and Andersson, B. 1969) (Sabolovic, D. and Dumont, F. 1973). Immunological activity is largely confined to the minor, cortisone-resistant subpopulation with

antigenic properties similar to peripheral T cells (Blomgren, H. and Andersson, B. 1969) (Takiguchi, T. et al 1971 b) (Schlessinger, M. 1972) (Konda, S. 1972) (Shortman, K. et al 1973). It is generally assumed that the TL-positive, high θ cells are the immature precursors of the low θ,TL-negative cells. An alternative model has been proposed, however, in which the prethymic cell can differentiate along one of two pathways. One pathway leads to a 'mature' but functionally blocked, high θ end cell, which may die within the thymus, and in the other a direct transformation to a low θ, TL-negative, immunocompetent thymus cell. In this model the high θ cells are not the immediate progenitors of low θ cells and peripheral T lymphocytes never possess the thymus-unique TL antigen (Schlesinger, M. 1972) (Shortman, K. and Jackson, H. 1974).

T cell heterogeneity also exists within the peripheral lymphoid tissues (Raff, M.C. and Cantor, H. 1971) (Bach, J-F. et al 1975 b). It is probable that at least a part of this heterogeneity can be explained by cells at different stages of maturation and experiments with thymic humoral factors have shed some light on this area. Two subsets of T cells have been described by Raff and Cantor, called T1 and T2 cells (Raff, M.C. and Cantor, H. 1971). T1 cells are located predominantly in the thymus and spleen, they decline in numbers rapidly (2 - 6 weeks) following adult thymectomy and bear large amounts of the θ isoantigen. T2 cells possess less θ antigen, are located in the lymph nodes and peripheral blood, are resistant to the short term effects of adult thymectomy but are sensitive to in vivo ALS treatment. A further T cell subset with little or no immunocompetence appears to exist. These cells have been termed 'post-thymic' by Stutman (Stutman, O. 1975) and T0 by Bach (Bach, J-F. et al 1975 b). These cells can be converted, under

the influence of a thymic humoral factor, to a population of more competent but still relatively immature, short-lived and non-recirculating (T1) cells. It has been suggested that the T0 - T1 transformation may be reversible and under the control of cAMP (Bach, M.A. et al 1975), which is itself regulated by thymic factors.

It is not known how many differentiation steps there are between the uncommitted haemopoietic stem cell and the mature, immunocompetent T cell. A hypothetical scheme showing five steps is depicted in figure 1.1. In the first stage the stem cell becomes committed to the T cell differentiation pathway and hence probably deprived of its other options. Where and how this takes place is not known. The process appears to be independent of the thymus as prethymic cells (also known as prothymocytes) have been found in the spleen and bone marrow of nude mice and in embryonic liver (Komuro, K. and Boyse, E.A. 1973 a). The second step is likely to be initiated by a thymic humoral factor or thymic inducer acting on the committed prethymic cell within, and possibly outside, the thymus and triggering its differentiation to an immature thymus cell. This transition involves changes in surface phenotype, characterized by the appearance of surface differentiation antigens coded by the sets of genes: Thy-1 (θ), TL, Ly-1, Ly-2/3, Ly-5, MSLA (Scheid, M.P. et al 1975). These changes can be induced in vitro within two hours by an active process not involving cell diffusion.

I. T cell antigen assay

Several groups have developed in vitro assays for thymic humoral factors based on cytotoxic assays using antisera specific for T cells

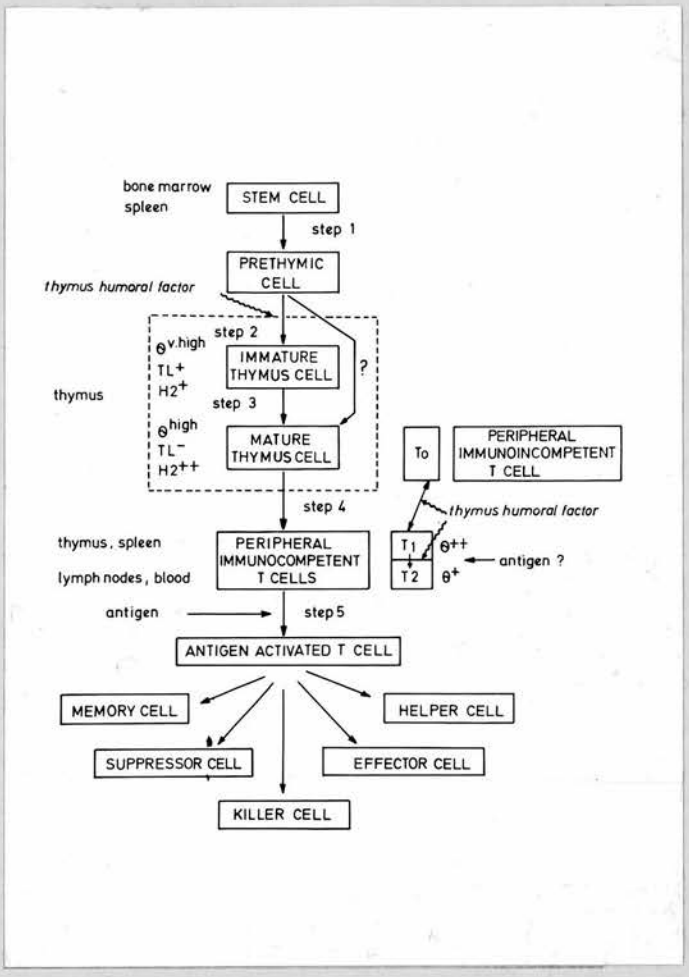


Figure 1.1. A scheme for T lymphocyte differentiation.

in mice (Komuro, K. and Boyse, E.A. 1973 a) (Komuro, K. and Boyse, E.A. 1973 b) (Scheid, M.P. et al 1973) (Wilson, O.H. and Bhaumick, B. 1973) (Basch, R.S. and Goldstein, G. 1974) (Miller, H.C. and Esselman, W.J. 1975) (Cohen, J.J. and Patterson, C.K. 1975) (Bach, M-A et al 1975), human (Touraine, J.L. et al 1974) and chicken (Teodorczyk, J.A. and Potworowski, E.F. 1975). In order to demonstrate the induction of T cell antigens the bone marrow or spleen cells were fractionated, usually by discontinuous density gradient centrifugation using bovine serum albumin (BSA). The cells sensitive to the inductive effect of thymic factors were found mainly in the lower density fractions. During early experiments with less purified mouse tissue extracts it was found that whilst thymic extracts were inducer-positive, the appropriate control tissue extracts were inducer-negative. It then became clear that a whole range of other substances, including spleen and other tissue extracts, cAMP, poly (A:U) and endotoxins could trigger, in vitro, the differentiation of T cell precursors (Scheid, M.P. et al 1973) (Scheid, M.P. et al 1975) (Singh, U. and Owen, J.J.T. 1975). It was found that, according to various metabolic criteria, cAMP was the mediator for the differentiation of prothymocytes (Scheid, M.P. et al 1975). This finding explained why, for example, epinephrine and β -adrenergic agonists, known to elevate cAMP in several tissues, were also effective as inducing agents. Scheid and co-workers also found that B cell differentiation was induced by cAMP, as demonstrated by the induction of various B cell markers such as the C'3 receptor (Scheid, M.P. et al 1975). Thymopoietin, a polypeptide thought to be specific to the thymus (Goldstein, G. 1974), was found to induce T cell but not B cell differentiation. UBIP, now called ubiquitin (Schlesinger, D.H. and Goldstein, G. 1975), on the other

hand was potent in inducing both T and B cell differentiation. The T cell and B cell precursors were thought to be different cells, each committed to their respective pathways, because of differences in the activity of thymopoietin and ubiquitin with respect to the β-adrenergic blocking agent propranolol and also because the T precursor cells were recovered from a denser cell fraction than immature B cells (Scheid, M.P. et al 1975).

It has been shown that precursor cells induced to differentiate into T cells under the influence of both a thymic extract and poly (A:U) were both functionally active as judged by their ability to co-operate in vitro with B cells in the production of antibody to sheep erythrocytes (Scheid, M.P. et al 1973). This was an important demonstration since it showed that functional maturation was correlated to changes in the cell surface.

Under normal physiological conditions, however, the thymus itself must act as the inducer since normal mice have cells with the phenotypic characteristics of T cells (θ, Ly antigens etc.) and athymic mice have not. Perhaps the small numbers of T cells which are occasionally found in nude mice might be explained by such differentiation.

II. Sheep erythrocyte rosette assay.

This assay is based upon the characteristic property of human thymocytes and T cells to form spontaneous rosettes with sheep erythrocytes (Wybran, J. et al 1972) (Jondal, M. et al 1972). Touraine and co-workers incubated fractionated human bone marrow cells with thymosin for two hours and found increased numbers of cells bearing T cell antigen, but little or no increase in the number of cells capable of forming rosettes with sheep erythrocytes (Touraine, J.L. et al 1974).

Incefy and co-workers, using longer incubation times, 2 - 14 hours, were able to detect increased numbers of rosette-forming-cells (RFC's) with human thymosin (Incefy, G. et al 1975). Both RNA and protein synthesis were found to be necessary for the induction of sheep erythrocyte reseptors. The longer incubation period needed in order to demonstrate the induction of sheep erythrocyte receptors as opposed to that required for the induction of T cell antigens was thought to indicate that the presence of sheep erythrocyte receptors was indicative of a more mature cell. A study made by Aiuti and co-workers (Aiuti, F. et al 1975) has confirmed the findings of Incefy.

Cells sensitive to the action of thymosin have been detected in the peripheral blood of normal humans following fractionation on BSA discontinuous density gradients (Vogel, J.E. et al 1975). These cells were different from those present in the bone marrow in that nucleic acid synthesis was not necessary for the induction of sheep erythrocyte receptors although protein synthesis was still required.

III. Sheep erythrocyte rosette inhibition assay.

Jean-Francois Bach and co-workers have developed a very sensitive assay for thymic factors based on the induction of θ antigen and measured by the amount of anti-θ serum or azathioprine needed to inhibit rosette formation with sheep erythrocytes (Bach, J-F. et al 1971 b) (Dardenne, M. et al 1973). Bach identifies three subpopulations of θ positive RFC's; To, T1 and T2 defined by rosette inhibition after in vitro incubation with anti-θ serum and complement.

To RFC's are inhibited only at high anti-θ serum concentrations, eg. (1/20) and are found in normal bone marrow. T1 RFC's are inhibited by low anti-θ serum concentrations, eg. (1/1000) and are present in the thymus and spleen. T2 RFC's are inhibited by low anti-θ serum

concentrations, eg. (1/200), although significantly higher than those acting on T1 RFC's, and are found in the lymph nodes and blood. For practical reasons azathioprine, a purine analogue, which can be obtained pure whereas anti-θ serum is a biological product of variable potency, is used. No differences have been found between the action of azathioprine and anti-θ serum. Thus, there is a similar ranking of azathioprine sensitivity from 1 μg/ml. for T1 RFC's, 50 μg/ml. for T2 RFC's to 500 μg/ml. for TO RFC's, expressed in minimum inhibitory concentrations (Bach, J-F. et al 1975).

Five days following adult thymectomy the sensitivity of the RFC's in the spleen of mice shows a sudden decrease corresponding to a loss of T1 RFC's (Bach, J-F. et al 1971 a). This decrease can be completely corrected by in vitro or in vivo treatment with small amounts of thymic extracts or of purified circulating "Thymic Factor" (Bach, J-F. et al 1971 b) (Bach, J-F. and Dardenne, M. 1973). The in vitro test involves incubating spleen cells obtained from adult mice, 10 - 20 days after thymectomy, with and without the thymic factor and with 10 μg/ml. azathioprine. This concentration of azathioprine is intermediate between that inhibiting spleen and normal thymus rosettes (T1 RFC's, 1 μg/ml.) and (T2 RFC's, 50 μg/ml.). After a 90 minute incubation at 37°C., sheep erythrocytes are added and the cell suspension centrifuged at 4°C. and gently resuspended. The rosettes are then counted in a haemocytometer. In the absence of a thymic factor, rosette formation is not inhibited by 10 μg/ml. azathioprine, however, in the presence of a thymic factor this azathioprine concentration inhibits the rosette formation of the newly induced T1 RFC's formed from the TO RFC's by the action of the thymic factor (Bach, J-F. 1975 c).

IV. In vitro graft-versus-host assay.

This procedure was originally developed by Auerbach and Globerson (Auerbach, R. and Globerson, A. 1966) and has been used extensively by Trainin and his associates in the study of the "thymus humoral factor" (THF). The relative enlargement of an F1 newborn spleen explant four days after challenge by parental lymphoid cells, compared to a paired explant challenged by F1 lymphoid cells, reflects the immune competente of the parental lymphoid cells. Preliminary experiments showed that spleen cells from neonatally thymectomized C57BL/6 young adult mice were deficient in the in vitro GvH assay compared to the same number of cells taken from unoperated C57BL/6 mice (Trainin, N. et al 1969). Competence was restored to the neonatally thymectomized spleen cells by either the continuous presence of THF over the four day culture or by a one hour preincubation with THF, followed by washing the cells free of THF. Exposure to antigen was therefore not required for the induction of immunocompetence. Incubation of mouse bone marrow cells with THF did not produce cells competent to perform in vitro GvH reactions. Competence was produced, however, if, after incubation with THF, the bone marrow cells were passaged through non-responsive mice and subsequently recovered from the spleen; or alternatively if the bone marrow cells were co-cultured with THF and fragments of spleen (Small, M. and Trainin, N. 1971).

In later experiments it was found that substances such as dibutyryl cAMP, poly (A:U), theophylline and PGE2, which increase the intracellular level of cAMP, were able to mimic the effect of THF and confer in vitro GvH reactivity to neonatally thymectomized mouse spleen cells (Kook, A.I. and Trainin, N. 1974). THF was found to act by

increasing adenyl cyclase activity and elevating the concentration of intracellular cAMP in thymus-derived cells. This finding substantiated the hypothesis that THF exerts its effect on non-immunocompetent spleen cells by inducing them to differentiate into more mature, immunocompetent cells. An early step in the activation process was thought to involve a rapid increase in intracellular cAMP. Protein synthesis occurred as a further step and if blocked, the acquisition of immunocompetence by spleen cells was abolished (Kook, A.I. and Trainin, N. 1975).

V. Mixed lymphocyte reaction.

Both THF (Trainin, N. et al 1975) and thymosin (Cohen, G.H. et al 1975) have been found effective in enhancing the reactivity of lymphoid cells to participate in one-way mixed lymphocyte reactions (MLR's). As both T1 and T2 cells are synergistically involved in the MLR (Asofsky, R. et al 1971) (Cohen, L. and Howe, M.L. 1973) the target cell, or cells, for activation by thymic extracts is not easily distinguished. Cohen found that the stimulating effect of thymosin was seen only using thymocytes and that the negative results for bone marrow and spleen cells might have been related to an inappropriate ratio of T1 to T2 cells (Cohen, G.H. et al 1975). It was suggested that, in the presence of thymosin and antigen T1 cells may be converted into T2 cells. More evidence is needed to substantiate this claim. The specificity of the enhancement in the MLR was uncertain as enhancing activity was found with spleen and other tissue extracts as well as with endotoxin (LPS). Another explanation for the effect of thymosin could be the proliferation of T2 cells during the 120 hour culture. Trainin found that a one hour preincubation of either spleen, lymph

node or thymus cells from intact mice, followed by washing the cells free of THF, enhanced their reactivity in the MLR. If THF was present during the response then a decreased response of effector cells was seen. PGE2 which has been shown to increase cellular cAMP levels (Kook, A.I. and Trainin, N. 1974) was found to mimic the effect of THF.

VI. In vitro mitogen assay.

In the last two or three years several assays for thymic factors based on increasing the responsiveness of lymphoid cells to T cell mitogens have been published. These reports are discussed in detail in this section.

The precise stage at which a cell undergoing T lymphocyte differentiation acquires the ability to respond to PHA is not known. Touraine found that, in the human, there was no increase in PHA or MLR response after a three hour incubation of fractionated bone marrow cells with thymosin followed by washing the cells (Touraine, J.L. et al 1974). In the same study a two hour incubation increased slightly the number of cells forming rosettes with sheep erythrocytes and increased significantly the number of cells sensitive to the cytotoxic property of an anti-T cell.serum. This indicated that responsiveness to PHA is acquired later in the differentiation pathway than the ability to form sheep erythrocyte rosettes and the possession of T cell antigens.

A preliminary report by Woody and co-workers suggested that the responsiveness of mouse bone marrow cells to PHA could be increased by prior incubation with a calf thymus extract (Woody, J.N. et al 1973). Bone marrow cells were incubated in medium containing 5% FCS and thymic extract for up to 16 hours, PHA was then added and the plates incubated

for a further 48 hours before tritiated thymidine was added and the cells harvested after a further 18 hours. Prior incubation for at least three hours was required before an increased response to PHA stimulation was found. Peak stimulation was seen after an 8 hour preincubation for normal C3H bone marrow cells but occurred earlier, after three hours, for thymectomized mouse bone marrow cells. Above-optimal concentrations of thymic extract were inhibitory and very little activity was reported for spleen extracts. All concentrations of the thymic extract were found to depress the response to PWM. The target cell for the thymic extract activity was not a cell that could be killed with anti-θ serum, as such treatment, although abolishing the response of the small pool of PHA-responsive cells normally present in the bone marrow, did not affect the results. The cells were not treated with anti-θ serum after the induction incubation and so it was not known whether the increased PHA response corresponded to the induction of the θ antigen.

THF has been reported to increase the reactivity of normal spleen cells to both PHA and ConA (Rotter, V. and Trainin, N. 1975). The increased response was found after the spleen cells had been incubated for 24 hours in medium containing 5% FCS and THF, and then washed free of THF, and cultured with PHA or ConA for a further 72 hours. The response to the mitogen was assessed by a two hour pulse of tritiated thymidine immediately prior to termination. THF did not modify the response to the B cell mitogen lipopolysaccharide (LPS), therefore indicating that the effect of THF was exerted on T lymphocytes only. A one hour preincubation with THF followed immediately by culture with PHA or ConA, in the absence of THF, neither increased nor decreased the response to the mitogen, Furthermore a one hour

preincubation with THF, followed by a 23 hour incubation without THF, before the addition of PHA or ConA also did not increase the mitogen response. A one hour incubation with THF had previously been found sufficient to increase the reactivity in the MLR (Trainin, N. et al 1975) (Umiel, T. and Trainin, N. 1975). The longer incubation time needed to increase mitogen responsiveness compared to other parameters of T cell differentiation was therefore consistent with the findings of others. When normal spleen cells were incubated with THF in the presence of ConA or PHA a reduction in the reactivity to the mitogen occurred compared to control cultures without THF. The response to LPS was not changed, however, therefore suggesting that the reduced reactivity of spleen cells to the T cell mitogens, in the presence of THF, was not the result of a toxic effect, but rather an expression of antagonism between THF and T cell mitogens in the activation of the T cell population. This was thought to be perhaps the result of an antagonistic effect between cAMP raised in the cells by THF (Kook, A.I. and Trainin, N. 1974) and cyclic guanidine monophopphate (cGMP) increased after exposure of cells to T cell mitogens (Hadden, J.W. et al 1972).

Thymopoietin has also been reported to induce responsiveness to T cell mitogens (Basch, R.S. and Goldstein, G. 1975 a) (Basch, R.S. and Goldstein, G. 1975 b). In order to detect increased responses to PHA and ConA spleen and bone marrow cells were fractionated on discontinuous BSA density gradients. This was necessary because increased responses to PHA and ConA were obscured by the pre-existing mitogen response of the unfractionated cells. Cells from the lower density fractions, normally unresponsive to T cell mitogens, became

responsive to ConA and PHA but not to LPS, following incubation with thymopoietin. No alterations were found in the response of fractionated adult thymus cells to PHA or ConA. The increase in the response to ConA in the spleen and bone marrow cells was always greater than the corresponding increase in response to PHA. This was not found by Rotter, who reported the reverse (Rotter, V. and Trainin, N. 1975). This was perhaps because in Rotter's assay young T cells were probably the predominant target, whilst in Basch's assay prethymic cells were the target for the action of the thymic factor. Rotter did not test this, however, by pretreating the cells with anti-θ serum. The time course for the induction of increased mitogen responsiveness during culture with thymopoietin was similar to that reported for the induction of T cell alloantigens (Basch, R. and Goldstein, G. 1974), with the capacity to respond to ConA reaching a maximum after six hours. The target cell for the activity of thymopoietin was shown to be a cell insensitive to the cytotoxic action of anti-θ serum. Treatment with anti-θ serum and complement after the induction incubation, however, abrogated the increased mitogen response showing that the cells acquiring the functional capacity to respond to PHA and ConA had also been induced to express the θ antigen.

A preliminary report by Serrou and co-workers suggested that an incubation with a crude thymic extract increased, by a factor of four, the response to PHA of an unfractionated normal mouse bone marrow cell preparation (Serrou, B. et al 1975). A control kidney extract showed no stimulating effect. Few other details were given except that the bone marrow cells were preincubated with 100 μg per ml. of extract.

A collaborative study by Bach and Hayry found that when nude mouse spleen cells were incubated with thymic factor (TF) for two hours

at 37°C and then washed and cultured with mitogen an increased response to ConA but a weaker and not increased response to PHA occurred (Bach, J-F. et al 1975 c).

Cercek and co-workers used fluorescence polarization to study changes in the PHA response of mouse bone marrow cells after incubation with a thymic extract (Cercek, L. et al 1975). These workers measured changes in the "structuredness of the cytoplasmic matrix (SCM)" as an index of increased DNA synthesis. For example, when peripheral blood lymphocytes were stimulated by mitogens or antigens a decreased SCM was observed. Normal mouse bone marrow cells, however, did not show a decreased SCM following stimulation by PHA. However, prior incubation for 10 - 200 minutes with a mouse or calf thymic extract (thymosin) rendered the cells responsive to PHA as judged by decreases in the SCM. Protein synthesis was found to be necessary for the induction of PHA responsiveness. Spleen extracts, PPD, endotoxin and DBcAMP had no effect in this assay. The authors thought that about 20% of the bone marrow cells were altered by treatment with thymic extract.

A recent report by Cohen and co-workers suggests that care should be taken in the interpretation of results which appear to imply that cells are induced by thymic extracts to show PHA or ConA responsiveness (Cohen, J.J. and Patterson, C.K. 1975). It was shown that bone marrow cells can be induced to express θ and TL antigens by ConA and PHA and then these cells can differentiate into lymphoblasts.

C H A P T E R T W O

Development Of A Method For Culturing Whole Blood And Lymphocytes With PHA.

OBJECTS

The object of this study was to develop and evaluate an in vitro test of thymic function. This test would then form the basis for monitoring attempts, both in vivo and in vitro, to reconstitute cell-mediated immune function in thymus-deprived rats.

It was decided to investigate the possibility of using a whole blood PHA culture technique to differentiate between normal and neonatally thymectomized (NTx) rats. This technique has the advantage of allowing the animal to survive and the reassurance that results from future tests done on the same animal are unmodified by prior antigenic stimuli. The previously widely used tests for assessing cellular immunity, for example allogeneic skin grafting, antibody formation to thymus-dependent antigens and dermal delayed hypersensitivity tests, all involve exposure of the test animal to foreign antigens, which means that results from subsequent tests done on the same animal, are influenced by the immunological memory to those antigens. Additionally, attempts were made to develop culture conditions allowing the measurement of the PHA response of lymphoid cells that had been isolated from the spleen, bone marrow and thymus.

INTRODUCTION

I. Phytohaemagglutinin and lymphocyte transformation.

Phytohaemagglutinin (PHA) is the name given to the family of glycoproteins obtained by aqueous extraction of the red kidney bean, Phaseolus vulgaris (Rigas, D.A. and Osgood, E.E. 1955). Landsteiner in the early 1900's discovered and subsequently did much work on the haemagglutinating property of PHA (Landsteiner, K. 1936).

PHA agglutinates the erythrocytes of all human blood groups, as well as those of the rabbit, dog, cat, chicken, mouse, rat, sheep, horse, pig, frog, guinea-pig, and to a limited extent, beef. (Technical information, Difco Laboratories, Detroit, Michigan). The haemagglutinating properties of Phaseolus extracts were used by Dorset and Henley to remove erythrocytes from a preparation of anti-Hog cholera serum (Dorset, M. and Henley, R.R. 1916). Li and Osgood similarly used PHA to prepare suspensions of leukocytes free from red blood cells (Li, J.G. and Osgood, E.E. 1949). PHA also has the property of agglutinating leukocytes in vitro (Naspitz, C.K. and Richter, M. 1968).

In 1959 Hungerford discovered the mitogenic property of PHA (Hungerford, D.A. et al 1959). When PHA was present in a culture of leukocytes, a marked transformation of mononuclear cells into large primitive cells (blast cells) capable of undergoing cell division was noticed after two and three days in culture. This result was soon confirmed by Nowell and other workers (Nowell, P.C. 1960) (Hastings, J. et al 1961) (Carstairs, K. 1962) (Marshall, W.H. and Roberts, K.B. 1963 a). The precursor cell of the blasts was found to be the small lymphocyte. It was soon discovered that other plant extracts, bacterial products, antibodies, endotoxins, allogeneic lymphocytes and many other substances were capable of initiating mitosis in cultured lymphocytes (Ling, N.L. 1968) (Oppenheim, J.J. 1968) (Naspitz, C.K. and Richter, M. 1968) (Hirschhorn, K. 1969). Many of the known lymphocyte activators can be classified into one of two types: specific or non-specific. Non-specific activators, for example PHA, Concanavalin A (ConA) and Pokeweed mitogen (PWM) stimulate a large proportion of lymphocytes from the peripheral blood of normal subjects. Specific activators (antigens), on the other hand, only stimulate lymphocytes obtained from

specifically immunised individuals. For example, the addition of tuberculin purified protein derivative (PPD) to cultures of lymphocytes from individuals sensitive to tuberculin induced the appearance of blast cells and cells undergoing mitosis (Marshall, W.H. and Roberts, K.B. 1963 b). The specificity of antigen-induced lymphocyte transformation in vitro was found to reflect the specificity of antigen-induced delayed skin reactions in vivo (Oppenheim, J.J. 1968). In contradistinction to PHA, which induces the enlargement of 70 - 90% of peripheral blood small lymphocytes by three days in culture, specific activators stimulate the enlargement of a small population 5 - 40%, of cells by five to ten days culture (Naspitz, C.K. and Richter, M. 1968). The reason for the time difference between the two responses is not known but it is perhaps related to the relative proportion of cells which are initially responsive. Much of the interest for this work derives from the observation that the gross morphological and biochemical characteristics of mitogen-induced lymphocyte transformation are very similar to antigen-induced immune reactions in vivo. It has been suggested that the various non-specific mitogens bypass the requirement for antigenic recognition and induce cells to undergo that pattern of response normally dependent upon immunological activation (Coulson, A.S. and Chalmers, D.G. 1964).

Within the past few years it has become apparent that the small lymphocyte population is heterogeneous and that a dichotomy exists between thymus-derived (T) cells and thymus-independent (B) cells (Roitt, I.M. et al 1969). Furthermore the T cell population (Raff, M.C. et al 1971) and the B cell population (Transplantation reviews volume 24 (1975)) are themselves heterogeneous. It was found that certain non-specific activators were able to stimulate lymphocytes from a particular class or classes only. The response of T and B cells

to various activators has been reviewed (Janossy, G. and Greaves, M.F. 1971) (Daguillard, F. 1972) (Daguillard, F. 1973). The apparent ability of PHA selectively to activate T cells has been used by many workers in studies of thymus-dependent immunity. The evidence for this assumption is reviewed in the following two sections.

II. Evidence for the selective activation of T cells by PHA.

The evidence for T cell involvement in the response to PHA can be presented under three general headings:

1. Experimental manipulations involving the selective depletion of lymphocyte populations.
2. Cell marker techniques.
3. Congenital or naturally acquired immunological deficiency.

1. Experimental manipulations involving the selective depletion of lymphocyte populations.

(A) Neonatal thymectomy

Neonatal thymectomy, in many species, has been shown to cause a persistent lymphopenia and reduced cell numbers in the spleen and lymph nodes. The decrease in total and relative T cell numbers has been associated with a decreased response to PHA, in mice (Martial-Lasfargues, C. et al 1966)(Dukor, P. and Dietrich, F.M. 1967) (Rodey, G.E. and Good, R.A. 1967) (Stutman, O. 1970) (Takiguchi, T. et al 1971 a) (Byrd, W.J. et al 1973), in rats (Rieke, W. 1966) (Meuwissen, H.J. et al 1969 a) (Dabrowski, M. et al 1970) and chickens (Greaves, M.F. et al 1968) (Meuwissen, H.J. et al 1969 b).

(B) Adult thymectomy plus irradiation

Adult thymectomy followed by total body irradiation, either sublethal or lethal, with bone marrow cell replacement therapy leads

to a deficit in the total number of T cells. This treatment has been shown to reduce PHA responsiveness in mice (Doenhoff, M.J. et al 1970) (Janossy, G. and Greaves, M.F. 1971) (Stockman, G.D. et al 1971) (Stobo, J.D. 1972) (Stobo, J.D. et al 1972), rats (Harding, B. et al 1971) (Penhale, W.J. et al 1973) and chickens (Alm, G.V. 1971).

(C) Adult thymectomy plus anti-lymphocyte serum.

Anti-lymphocyte serum (ALS) treatment, following adult thymectomy, is a procedure that has been shown permanently to reduce the animal's T cell population. This treatment has been shown to reduce the response of mouse peripheral blood leukocytes to PHA (Tursi, A. et al 1969).

(D) Thoracic duct drainage.

Recirculating lymphocytes (predominantly thymus-derived), obtained from the thoracic duct of rats, were found to be PHA-responsive, whilst non-recirculating lymphocytes, obtained from the blood of rats after three days of thoracic duct drainage, were poorly responsive to PHA (Iversen, J-G. 1969).

(E) Bursectomy.

Chickens, either chemically or surgically bursectomized, whether having received total body irradiation or not, whilst showing reduced numbers of B cells and poor antibody responses did not show reduced PHA responses (Greaves, M.F. et al 1968) (Alm, G.V. and Peterson, R.D.A. 1969) (Meuwissen, H.J. et al 1969 b) (Kirchner, H. et al 1973).

2. Cell marker techniques.

(A) Chromosomal.

i. T6T6: Chromosome analysis of cells dividing in response to PHA in mice that had been thymectomized, lethally irradiated,

injected with bone marrow cells and reconstituted with a T6T6 thymus (cells of T6T6 origin are distinguishable by means of two marker chromosomes in metaphase preparations) showed considerable numbers of dividing cells of thymus graft origin (Doenhoff, M.J. et al 1970).

ii. Sex: Rats thymectomized at 5 - 7 weeks and three weeks later given 900 rads total body irradiation, were reconstituted with isologous bone marrow cells, and thymus cells of the opposite sex. Peripheral blood cultures subsequently showed that approximately 90% of the cells dividing in response to PHA came from the innoculum of thymus cells (Johnson, J.M. and Wilson, D.B. 1970).

(B) Anti-T cell serum.

Treatment of lymphoid cells with an anti-T cell serum and complement both reduced the number of T cells and the response to PHA in man (Aiuti, F. and Wigzell, H. 1973) (Bobgrove, A.M. et al 1974) and in rats (Balch, C.M. and Feldman, J.D. 1974). In more specific terms, mouse T cells possess the surface marker Thy-1(θ) which is not present on B lymphocytes (Raff, M.C. 1970). Treatment of lymphoid cells with anti-θ serum and complement specifically eliminates T cells. This procedure was found to reduce drastically the PHA responsiveness of mouse lymphocyte populations (Janossy, G. et al 1971) (Blomgren, H. and Svedmyr, E. 1971) (Owen, J.J.T. et al 1971) (Howe, M.L. 1973).

3. Congenitally or naturally acquired immunodeficiency.

(A) Cell-mediated.

i. Man: Lymphocytes from the peripheral blood of patients with thymic aplasia (DiGeorge's syndrome) show a markedly reduced

response to PHA (Lischner, H.W. et al 1967) (Oppenheim, J.J. 1968) (Gotoff, S.P. 1968) (August, C.S. et al 1968) (Cleveland, W.W. et al 1968) (Kretschmer, R. et al 1968) (Kazimiera, J. et al 1973).

ii. Mouse: The lymphoid tissue of congenitally athymic, nude mice have been shown to be deficient in T cells (Raff, M.C. and Wortis, H.H. 1970). Lymphocytes from these mice were found to be unresponsive or very poorly responsive to PHA (Janossy, G. et al 1971) (Schuman, G. et al 1973) (Thurman, G.B. et al 1975).

(B) Humoral, for example agammaglobulinaemia.

The majority of investigators have reported that, in the case of selective B cell defects, such as impairment of humoral immunity or primary agammaglobulinaemia, congenital or acquired, peripheral blood lymphocytes incubated with PHA showed a degree of blast transformation comparable to normal (Fudenberg, H.H. and Hirschhorn, K. 1964) (Ripps, C.S. and Hirschhorn, K. 1967) (Bradley, J. and Oppenheim, J.J. 1967) (Cooperband, S.R. et al 1968 b) (Gotoff, S.P. 1968) (Lieber, E. et al 1969).

III. The response of B cells to PHA.

The results quoted in the previous section, directly and indirectly, implies that the T cell is PHA-responsive and that the B cell responds poorly or not at all. Direct proof concerning the responsiveness of the B cell population towards PHA will only be obtained when pure populations of T and B cells can be prepared. This entails the developmant of techniques for the identification and separation of T and B cells. The most used markers for T cells are the

θ antigen, for murine cells (Raff, M.C. 1971) and the ability to form E rosettes with sheep erythrocytes (Wybran, J. et al 1972) (Jondal, M. et al 1972). B cells of several species have been shown to possess a high density of surface immunoglobulin (Ig) (Pernis, B. et al 1970) (Raff, M.C. 1971) (Unanue, E.R. et al 1971) (Wilson, J.D. and Nossal, G.V.J. 1971). B cells also carry a receptor for activated complement factor 3 (Bianco, C. et al 1970) (Dukor, P. et al 1971), and will bind Ig via the Fc piece if the Ig belongs to certain subclasses and is complexed with antigen (Basten, A. et al 1972).

Pure T and B cell populations can be prepared more easily from animals than from human lymphocytes. Relatively pure B cells can be obtained from the spleens of mice, thymectomized when adult, lethally irradiated and reconstituted with bone marrow cells pretreated with anti-θ serum and complement. Any residual θ -positive cells can be removed by incubating the separated spleen cells with anti-θ serum and complement (Elfenbein, G.J. and Gelfand, M.C. 1975). The congenitally athymic, nude mouse (Pantelouris, E.M. 1968), which is freely available, can also be used as a donor of B cells. Pure populations of T cells can be obtained from the thymus and cortisone-resistant T cells can be obtained from the thymus two or three days after an injection of cortisone acetate (Elfenbein, G.J. and Gelfand, M.C. 1975). One method for the separation of human lymphocytes makes use of the ability of T cells to form rosettes with sheep erythrocytes. T cells, when bound to sheep erythrocytes are separated from non-rosetting lymphocytes (B cells) by using a ficol-hypaque gradient, the rosetting cells being collected in the pellet and the non-rosetting cells at the interphase, This procedure was found to be highly effective, the preparations of non-rosetting cells contained less than

1% rosetting lymphocytes; less than 2% of the rosetting lymphocytes carried detectable amounts of surfage Ig (Lohrmann, H-P. et al 1974). BSA gradient centrifugation has also been used in conjunction with sheep erythrocyte rosetting as a means of preparing T and B cell populations (Geha, R.S. et al 1974). Column immunoabsorption techniques have also been used to separate T and B cells. For example, Chess and co-workers used a sephadex G-200 column bound with a pure rabbit anti-human Fab preparation (Chess, L. et al 1974) and Phillips used a column bound with a rabbit anti-human light chain globulin (Phillips, B. and Roitt, I.M. 1973).

Using the above and similar approaches it has been claimed that under "normal" culture conditions:

a. Pure B cell populations do not respond to PHA (Geha, R.S. et al 1974) (Greaves, M.F. et al 1974) (Lohrmann, H-P. et al 1974) (Piguet, P-F. and Vassalli, P. 1972),

b. In mixed T/B cell cultures, B cells do respond to PHA (Vischer, T.L. 1972) (Piguet, P-F. and Vassalli, P. 1972) (Phillips, B. and Roitt, I. 1973) (Elfenbein, G-J. et al 1973) (Geha, R.S. et al 1974) (Greaves, M.F. et al 1974) (Epstein, L.B. et al 1974) (Chess, L. et al 1974) (Phillips, B. and Weisrose, E. 1974) (Lohrmann, H-P. 1974).

That B cells respond to PHA in mixed T/B cell cultures was shown by the following techniques. Phillips and Roitt used a rabbit anti-human light chain serum as a marker for surface Ig on human lymphocytes. After incubation with a fluorescein-conjugated goat anti-rabbit antiserum stained blast cells were visualised under ultra-violet light (Phillips, B. and Roitt, I.M. 1973). Evidence was produced to show that the staining of the blasts was not due to a T cell membrane component nor

was it due to passive adsorption of Ig from B cells in culture. However it was possible that the Ig was adsorbed onto T cell blasts by close cell contact. Elfenbein and co-workers identified proliferating B cells by using, simultaneously, tritiated thymidine incorporation and autoradiography, and rosette formation with sheep erythrocytes bearing activated third component of complement (C3) on their surface (Elfenbein, G.J. et al 1973). The possibility that activated T cells could acquire a receptor for C3 had been ruled out by experiments in which mouse thymocytes and cortisone - resistant thymocytes had been cultured with or without ConA for 48 hours. At the end of this peripd the lymphocytes were incubated with sheep erythrocytes as above, and no rosettes were observed. It was still possible, however, that in mixed T/B cell cultures C3 receptors could have been adsorbed onto T cell blasts. In a study using mouse radiation chimeras containing chromosomally marked T cells, chromosome analysis of cells arrested at metaphase, after culture with PHA or allogeneic lymphocytes, demonstrated B cell mitoses (Piguet, P-F. and Vassalli, P. 1972).

A critical early step in lymphocyte activation is the binding of the phytomitogen to specific sites on the cell membrane (Lindahl-Kiessling, K. and Peterson, R.D.A. 1969) (Weber, T.H. and Lindahl-Kiessling, K. 1972) (Greaves, M.F. et al 1972). Since PHA and ConA bind to T and B cells in approximately equal numbers (Stobo, J.D. et al 1972) (Greaves, M.F. et al 1972) (Möller, G. et al 1973) the failure of B cells to bind mitogen is not the reason for the lack of response of pure populations of B cells. Two possible mechanisms to explain B cell proliferation to soluble PHA and ConA in mixed T and B cell cultures are: (1) Mitogens bound to T cells or other cell types may

stimulate B cells to proliferate; (2) T cells stimulated by mitogen may elaborate specific or non-specific factors that stimulate or facilitate B cell proliferation. Experimental evidence exists to support both mechanisms. There may also be other mechanisms besides the two mentioned.

Pure mouse B cell populations have been stimulated to proliferate by insolubilized ConA covalently linked to the bottom of a plastic petri dish (Andersson, J. et al 1972 b) (Möller, G. et al 1973). Similarly spleen cells from mice which had been thymectomized, lethally irradiated and reconstituted with bone marrow cells that had been pretreated with anti-θ serum and complement, were responsive to PHA covalently bound to sepharose beads (Greaves, M.F. and Bauminger, S. 1972). Almost pure populations of mouse B cells have been shown to be directly stimulated by PHA and ConA bound onto the surface of thymus cells, which themselves could not respond because of prior treatment with mitomycin C and cycloheximide (Elfenbein, G.J. and Gelfand, M.C. 1975). The small numbers of contaminating T.cells in the B cell preparation were shown to have little or no role in the response of the B cell population to mitogen-bound,pulse-poisoned thymocytes. The release of mitogen from the mitogen-pulsed thymocytes was shown to be in amounts that did not cause stimulation by soluble mitogen. In cultures using mitogen-pulsed, non-poisoned thymocytes, the increased B cell proliferation due to the release of possible blastogenic factors from the thymocytes was thought to be of minor importance. It was also found that mitogen, bound to B cells, was capable, though not to the same extent as mitogen bound to T cells, of stimulating B cells. The authors concluded that in mixed populations of T and B cells, mitogen bound to T cells plays the major role in the stimulation

of B cells (Elfenbein, G.J. and Gelfand, M.C. 1975).

There is also evidence to support the idea that, in response to activation by PHA, ConA and antigens, soluble mediators are released, enabling B cells to respond to the stimulants. ConA is a mitogen thought to be specific for the activation of T cells (Andersson, J. et al 1972 c).(Janossy, G. and Greaves, M.F. 1972). Mouse B lymphocytes, either spleen cells from nude mice or from adult thymectomized, lethally irradiated mice reconstituted with anti-θ serum-treated bone marrow cells, were found to be able to respond to ConA when cultured in either undiluted or half-diluted supernatants obtained from a normal thymus cell culture (Andersson, J. et al 1972 a). A factor present in the supernatant of a 24 hour culture of human peripheral blood lymphocytes and ConA was found to be mitogenic in cultures of both autologous and allogeneic lymphocytes (Smith, J.L. and Barker, C.R. 1972). This activity was shown not to be due to a "carry-over" of mitogen. The stimulation of mouse B cells in the presence of PHA-activated T cells has been ascribed as due to a humoral factor released from activated T cells (Piguet, P-F. and Vassalli, P. 1972). Piguet found that the factor appeared within 24 hours in the supernatant of a mouse mixed lymphocyte culture (MLC) and that this factor, though not itself mitogenic for B cells, allowed the proliferation of B cells in the presence of PHA. However, human T cells during culture with PHA have been shown not to release such a factor (Geha, R.S. et al 1974) (Epstein, L.B. et al 1974). In the case of the in vitro stimulation with antigen, to which the donor cells were immune, it was found that human B cells did not transform with antigen alone but transformed vigorously in the presence of supernatants

derived from antigen-activated T cells (Geha, R.S. et al 1973) (Geha, R.S. and Merler, E. 1974).

In summing up, whilst the mechanism of B lymphocyte proliferation in response to PHA is still unclear, the extent of B cell participation using standard culture techniques would appear to be minimal, especially during the first three days of culture. A distinctive feature of the B cell response is that it appears to occur later in culture than that due to T cells.(Piguet, P-F. and Vassalli, P. 1972) (Epstein, L.B. et al 1974) (Chess, L. et al 1974). The PHA response can therefore be considered, under controlled conditions of cellular composition, culture medium and assay to be a semi-quantitative measure of the capacity of T cells to become activated and divide (Greaves, M.F. et al 1974).

IV. Techniques for lymphocyte culture and assessment of the response to PHA

Conventional techniques for lymphocyte culture require the isolation of lymphocytes from a relatively large volume of blood (Hughes, D. and Caspary, E.A. 1970), or the availability of lymphoid organs from which a cell suspension can be prepared. Whole blood culture has several advantages which include :- (a) a decrease in the volume of blood required to carry out the same number of cultures. This means that small laboratory animals can be tested several times with minimum interference with their metabolism. (b) there is no need for complicated and time consuming lymphocyte separation procedures. A disadvantage of whole blood techniques is that direct comparison of the response of different subjects is complicated by differences in the total cell number in samples of whole blood. However, comparison

of such responses perhaps more accurately reflects immune competence.

Several whole blood microtechniques have been developed for the culture of human blood (Araki, D.T. and Sparkes, R.S. 1963) (Junge, U. et al 1970) (Park, B.H. and Good, R.A. 1970) (Pauly, J.L. and Sokal, J.E. 1972) (Paty, D.W. and Hughes, D. 1972) (Pauly, J.L. et al 1973) (Luquetti, A. and Janossy, G. 1976). Whole blood techniques have also been applied successfully to laboratory animals (Han, T. and Pauly, J.L. 1972) (Heiniger, H.J. 1973).

The degree of lymphocyte transformation can be assessed by changes in lymphocyte morphology or by changes in the rate of macromolecular synthesis. After about 36 hours in culture with PHA, blast cells begin to appear. These are large cells with a large nucleus and a deeply basophilic cytoplasm, which can divide by mitosis. The number of blast cells, or cells arrested at metaphase following the addition of colchicine to the culture, counted in a standard number of cells (at least 200) gives a measure of the lymphocyte response to that particular activator. Many metabolic alterations occur in lymphocytes undergoing blastogenesis. Through the use of radioactively-labelled precursors, the synthesis of protein, DNA and RNA has been used as a measure of the proliferative response to PHA (Ling, N.L. 1968). The labelled cells are either counted autoradiographically or the total radioactive uptake is measured by liquid scintillation spectro-photometry. The most frequently used method for quantifying the response to PHA is by measuring the rate of DNA synthesis using the incorporation of a labelled precursor such as tritiated thymidine. This method, and others, is far removed from the initial activation of responsive lymphocytes and the results can be complicated by cell death, repeated cell division and by the release of factors which might modify the response. The ideal assay, based on an early irreversible step in the activation process and being rapid, precise and practical has not yet been developed.

MATERIALS AND METHODS.

This section will describe the materials and the methods used during the development of the lymphocyte culture system.

MATERIALS.

1) Tissue culture media.

   a) Eagle's basal medium (EBM), single strength.

   b) Eagle's minimum essential medium (EMEM), (x10) concentrate.

   c) TC199, single strength and (x10) concentrate.
      Wellcome Research Laboratories,
      Beckenham, England.

   d) RPMI 1640, single strength.
      FLOW Laboratories, Ltd.,
      Irvine, Scotland.

2) Medium supplements.

   a) Sterile sodium bicarbonate,
      Pharmaceutical Dept.,
      Edinburgh Royal Infirmary.

   b) Antibiotics, "Crystamycin".
      Glaxo.

   c) l-glutamine (200 mM),
      FLOW Labs. Ltd.

   d) HEPES (1M)
      FLOW Labs. Ltd.

   e) Foetal calf serum, (FCS). (not heat-inactivated).
      Wellcome Research Laboratories.

3) Bacto-Phytohaemagglutinin-P,
   Difco Laboratories,
   Detroit, Michigan.

4) Heparin, 'Pularin', 5,000 units per mL; pyrogen-free, preserved with 0.15% chlorocresol.

Evans Medical Ltd.,

Speke, Liverpool.

5) Tritiated thymidine,

The Radiochemical Centre,
Amersham.

6) Thymidine,

Sigma.

7) Trichloroacetic acid (TCA), AnalaR,

BDH Chemicals Ltd.

8) Methanol,

BDH Chemicals Ltd.

9) Hyamine hydroxide (iM in methanol),

Nuclear Enterprises Ltd.,

Sighthill, Edinburgh 11.

10) Scintillation fluid, Liquifluor.

New England Nuclear, NEN Chemicals,

18 Glencross Gardens, Penicuik, Midlothian.

(4.0 g, 2,5 diphenyloxazole (PPO), 50 mg 1,4-bis-2-(5-phenyl-oxazolyl)-benzine (POPOP) and 20 ml absolute ethanol made up to 1 litre with toluene).

11) Toluene (Scintillation grade),

BDH Chemicals Ltd.

12) Culture vessels:

a) Staynes, plastic, flat-bottomed, screw-capped,

50 mm x 13 mm, 5 ml capacity vials, sterile.

b) Sterile universal containers, 90 x 24 mm., 30 ml. capacity, polystyrene/polypropylene,

Sterilin.

c) Sterile bijou, 50 x 18 mm., 7 ml. capacity, polystyrene/polypropylene,

Sterilin.

13) Eppendorf pipettes: 1,000, 500, 200, 100, 50, µl.

14) Airtight polythene box, approximately 25 x 19 x 12 cm. with added gas inlet, and outlet ports.

15) Scintillation counting vials, screw-neck, low potassium.

FBG Trident, Ltd.

16) Lithium heparin tubes 10 ml. capacity.

TEKLAB.

17) Sterile, plastic disposable syringes.

PLASTIPAK.

METHODS

I. Animals and operative techniques.

Animals

Male and female Sprague-Dawley rats of a highly inbred strain were supplied by the Animal Diseases Research Institute (ADRI), Edinburgh. These rats were bred within the department of Clinical Surgery for three generations only, when a new foundation stock was obtained from ADRI. The rats were fed standard rat cake* and water

* Macgregor and Co. (Leith) Ltd., Quayside Mills, Leith, Scotland.

ad libitum.

Rats used to donate blood and lymphoid organs for the in vitro studies were usually aged between 40 - 80 days. However, in a number of cases older animals were used, these instances are indicated in the text. Animals used to provide serum or blood for ethrocyte preparations were often older than 80 days. Most of the in vitro studies were done using tissues from male rats, however, in a few cases female donors were used.

Thymectomy and sham thymectomy

Thymectomy (Tx) or sham thymectomy (STx) was performed on rats within 72 hours of birth. The operation was done, under ether anaesthesia, by a suction method through a longitudinal, trans-sternal incision. The wound was closed by one suture. Sham thymectomy was carried out using exactly the same technique except that after the sternum had been divided, to expose the thymus, the wound was closed. After initial failures operative mortality was low (less than 10%). The greatest threat to the life of the operated rats was parental neglect and cannabalism and sometimes whole litters were lost in this way. Rats were weaned at about three weeks of age and any runted animals were killed. Only healthy rats were used in experiments. There was no sign of the post-thymectomy wasting syndrome. Thymectomized rats gained weight at the same rate as intact rats, see figure 4.9, and survived for at least one year when all unused experimental animals were killed.

Thymus grafting

In the first thymus reconstitution experiment (chapter four) the grafts were placed subcutaneously. Recipient rats were

anaesthetized and a subcutaneous pocket prepared on the back of each rat. Two thymuses from freshly killed neonatal donor rats were cut into several pieces and inserted under the skin, using a pair of forceps. The pocket was then closed with a single suture. In the second and subsequent experiments the grafts were placed under the kidney capsule. The left kidney of the rat was exposed through an incision in the abdominal wall. The donor thymuses were cut into fragments in cold saline and drawn into the end of a plastic cannula (inside diameter 1.4 mm.), connected to a 2 ml. syringe. A hole was made in the kidney capsule and the cannula carefully inserted and by gentle pressure the thymus fragments were inserted flush to the kidney. In some instances pieces of thymus were also placed in the fat surrounding the kidney. The abdominal wall was then closed with two silk 2-0 sutures and the skin closed with three of four sutures.

Post-mortem procedures.

Each thymectomized or thymus grafted rat was carefully examined, shortly after death, for the presence of a thymus remnant or thymus graft. Any suspicious tissue was removed, fixed in formol saline and standard haematoxylin and eosin stained paraffin sections prepared. Thymectomized rats found to possess a thymus remnant were either excluded from the results or are referred to individually in the text.

II. Preparation, heat-inactivation and storage of serum.

Fresh human, rat or rabbit blood, containing no anticoagulant, was placed in a sterile glass centrifuge tube and left at 4°C. for a minimum of one hour, and at most overnight. The clot was then freed from the side of the tube and the blood was centrifuged at

3,000 rpm. for 15 minutes, at room temperature, in a MSE minor centrifuge. The serum was then transferred to a sterile plastic vial using a Pasteur pipette and was either used within 24 hours of stored at -20°C until used.

Uninactivated foetal calf serum, obtained frozen, was allowed to thaw and was aliquotted in 5 ml. or 7 ml. amounts and stored at -20°C in sterile plastic or glass vials. Heat-inactivation of serum was carried out by placing the serum, contained in sterile glass bijous in a water bath at 56°C for 30 minutes. For one preparation only, heat-inactivation was carried out by placing the serum in an air incubator at 60°C for 30 minutes.

III. Reconstitution and storage of antibiotics.

The contents of the vial were made up to 5 ml. by the addition of sterile water. 0.5 m. aliquots were placed into sterile plastic vials and stored at -20°C until used. The concentration of the stock antibiotic solution was as follows:

Sodium benzylpenicillin - 100,000 units/ml.

Streptomycin sulphate - 100 mg/ml.

IV. Storage of l-glutamine (200mM).

The glutamine was allowed to thaw at room temperature and aliquots, slightly greater than 4 ml. were placed in sterile plastic vials and stored at -20°C until needed.

V. Storage of HEPES (1M).

Aliquots of a little more than 1 ml. were stored in sterile plastic vials at 4°C.

VI. Preparation and supplementation of media.

The single strength media contained:

Sodium bicarbonate: 110 mg/100 ml.

l-glutamine: (RPMI 1640) none

(TC199) 10 mg./100 ml.

(EBM) 29.2 mg/100 ml.

Antibiotics: Penicillin - 20,000 units/100 ml.

Streptomycin - 10,000 µg./100 ml.

The medium concentrates (x10) contained none of the above compounds. During the reconstitution of the medium concentrate with sterile distilled water the following supplements were added:

Sodium bicarbonate ; 125 mg./100 ml.

l-glutamine: 58.4 mg./100 ml.

Antibiotics: 20000 units Penicillin/100 ml.

20,000 µg. Streptomycin/100 ml.

In some cultures the medium was supplemented with HEPES by adding 1 ml. of HEPES (1M) to 99 ml. medium bringing the concentration of HEPES to 10 mM.

When serum was added to the culture medium its concentration was expressed in terms of the volume added as a percentage of the final volume.

VII. Reconstitution, storage and use of PHA-P.

The contents of the vial were dissolved by the addition of 5 ml. sterile physiological saline. Aliquots of 0.4 or 0.5 ml, in sterile plastic vials, were kept at -20°C until used. When required, a vial was thawed and a further dilution made, using sterile saline, such that a designated amount (between 0 - 25 µl) of the original 5 ml.

was present in 100 μl. of solution. 100 μl amounts of the diluted PHA solution, or saline, were then added to the culture vials as indicated in the text. No batch of PHA was used if longer than six months had elapsed since reconstitution.

VIII. Storage and use of tritiated thymidine.

Compounds of high and low specific activity were used:

High specific activity: Thymidine-6-(^{3}H),

sp. act. 21-27 Ci/m.mol.

Low specific activity: Methyl-(^{3}H)-Thymidine,

sp. act. 5.0 Ci/m.mol.

The high specific activity compound was obtained from Amersham in 250 μCi. amounts as an aqueous solution and was stored at a concentration of 250 μCi. per ml. of saline at -20°C. The low specific activity compound, part of a 5 MCi batch, stored in liquid nitrogen, was a gift from Dr. J. Masters, Department of Clinical Surgery, 300 μCi. amounts of this compound, in 1.2 ml. saline, were stored at - 20°C.

Immediately prior to use the compound was diluted in culture medium so that 1 μCi. could be added in a volume of 100 μl. The amount of tritiated thymidine added to each vial in culture was a standard 1 μCi.

IX. Preparation of lymphoid cells.

(a) Whole blood.

Rats were bled either from the heart or the aorta, humans were bled by venepuncture. Blood was obtained using sterile, plastic, disposable syringes which had been lightly heparinized and usually, 21 gauge microlances. The blood was gently mixed and carefully transferred to sterile plastic vials before addition to culture.

(b) Washed blood.

Whole blood, obtained as described above, was transferred to a Lithium heparin tube. The blood level was marked on the side of the tube which was centrifuged five times at 2,000 rpm for ten minutes. After each centrifugation, the supernatant was discarded and replaced with either medium or physiological saline and the blood was carefully remixed. Finally the washed blood was transferred to a sterile plastic vial.

(c) Spleen cells.

Spleens were removed using aseptic technique from ether-killed rats, some of which had been exsanguinated. The spleen was then washed in medium. Two methods for disrupting the spleen were used. In the earlier experiments the spleen was cut into very small pieces using sterile scissors; in later preparations the spleen was homogenized in medium using a loose-fitting, sterile glass homogenizer. The supernatant, about 10 mls., was transferred to a sterile plastic universal container, the debris allowed to settle for about 30 seconds before the supernatant was transferred to sterile glass centrifuge tubes. A cell count was then performed, and, in some early cultures if this count was satisfactory the cells were used without further treatment. In later cultures, and in early cultures if the cell concentration was not satisfactory, the cells were centrifuged at 1500 rpm for 10 minutes, all the supernatant discarded and the cells resuspended, in fresh medium by gentle suction in and out of a Pasteur pipette.

(d) Thymus cells.

The thymus was removed aseptically from a freshly killed rat

and the surrounding lymph nodes and much of the capsule removed. A cell suspension was prepared using the method described for spleen cells.

(e) Bone marrow cells.

Both femora were removed from each rat, cleaned of adhering flesh and washed in sterile medium or saline. Both ends of the femur were then cut off and the contents of the marrow cavity flushed out in 5 ml. medium using a syringe and a 21 gauge needle. The contents from two or more femora were pooled and a supernatant obtained by gravity sedimentation which was further treated exactly as described for spleen cells.

X. Total and differential white blood cell counts.

Total white cell counts, in blood and cell suspensions, were made on, usually, 1 in 10 or 1 in 20 dilutions of the test solution. The diluting fluid was 1% acetic acid tinged with methyl violet. The cells were counted in an improved Neubauer chamber.

Differential counts were made on Leishman-stained blood smears. At least 200 cells were classified as one of three cell types, lymphocyte, polymorphonuclear leukocyte or monocyte. If very few white cells were present, as was the case in blood samples from some thymectomized rats, less than 200 cells were counted.

XI. Viability of lymphoid cells.

The method of trypan blue dye exclusion (Boyse, E.A. et al 1962) was used to assess cell viability. A 0.1 ml. aliquot of the lymphoid dell suspension was added to 0.1 ml. of a 0.4% stock solution of trypan blue in saline. After incubation for 10 minutes at room

temperature, the number of viable (non-stained) cells was counted in a haematocytometer within 5 minutes after incubation. This test was performed routinely during early cultures when the cell viability was found to be always greater than 85% and often greater than 95%.

XII. Preparation of erythrocytes.

Whole blood was obtained from a thymectomized rat and transferred to a heparin tube. The level of blood was marked on the side of the tube. The blood was either allowed to sediment at room temperature for at least one hour, or was centrifuged, at 1,000 - 1,500 rpm for 5 minutes. The plasma and buffy coat leukocytes were then removed and medium or saline was added back. The cells were centrifuged twice more, at 1,500 rpm with the buffy coat and plasma removed each time. A further three centrifugations at 2,000 - 2,500 rpm were made to ensure that practically all the plasma was removed. After the final replacement with saline or medium the preparation was transferred to a sterile plastic vial and a total white cell count was performed.

XIII. Method of culture and assessment of the response to PHA by tritiated thymidine incorporation.

Staynes 5 ml. plastic screw-capped vials were used in all but two cultures when Sterilin 7 ml. plastic bijous were used. There was no apparent difference between the results obtained from each type of culture vessel. All manipulations were done in a double laminar flow air bench* and strict sterile technique was used. Additions to the culture vials were made using Eppendorf pipettes and autoclaved plastic disposable pipette tips.

The basic whole-blood culture technique was performed as follows:

* Slee, London.

1.0 ml. of medium (usually EBM or EMEM), 0.1 ml. of PHA in saline, or 0.1 ml. saline, and 0.1 ml. of whole blood were dispensed into each culture vial. The vials were gently shaken and placed, loosely-capped, into the polythene box which was then sealed with two layers of autoclave tape. The box was gassed with 5% CO_2 in air and incubated at 37°C for three days. Each day the atmosphere was renewed and the vials were gently shaken. After 48 hours in culture 1 μCi of (^{3}H) thymidine in 100 μl. of medium was added to each vial and incubation was continued for a further 24 hours. The contents of each vial were then transferred to a 10 ml. glass centrifuge tube with 5 ml. cold phosphate-buffered saline (PBS). The extent of DNA synthesis was determined by measuring the incorporation of tritiated thymidine into a trichloroacetic acid (TCA) precipitate. The procedure for obtaining the acid-insoluble precipitate is summarized in table 2.1. Centrifugations were done at room temperature using MSE Major and Minor centrifuges. The erythrocytes were lysed by vigorously mixing the precipitate with 3% acetic acid. This process has been shown to cause no loss of labelled DNA (Junge, V. et al 1970). The remaining brown pigment was removed during dehydration with two washes of absolute methanol. These procedures eliminated or considerably reduced colour quenching which would have occurred during liquid scintillation counting. Following the advice of Paty and Hughes (Paty, D.W. and Hughes, D. 1972) the TCA precipitate was allowed to form overnight at 4°C as short treatments with TCA were found insufficient to bring down all the labelled material (Paty, D.W. and Hughes, D. 1972). The final pellet was solubilized by the addition of 0.5 ml. of Hyamine hydroxide and incubation in sealed tubes at 37°C for 24 - 48 hours.

Table 2.1 Preparation of TCA precipitate from PHA-stimulated whole blood culture.

Step	Treatment	Centrifugation	Day
1.	5 ml. PBS	500 g.	one
2.	5 ml. 3% acetic acid	500 g.	one
3.	5 ml. PBS	500 g.	one
4.	5 ml. 5% TCA		one
		600 g.	two
5.	5 ml. 5% TCA	600 g.	two
6.	4 ml. methanol	600 g.	two
7.	4 ml. methanol	600 g.	two
8.	0.5 ml. Hyamine	-	two

The dissolved products were transferred to liquid scintillation counting vials using two 5 ml. volumes of toluene scintillation fluid. After several hours storage at 4°C, the radioactivity in each vial was counted over a ten minute period, in a refrigerated Nuclear Chicago Mark I liquid scintillation counter. The vials were counted several times until successive stable counts were obtained. Replicate counts tended to decrease slightly before becoming stable. The results were derived from the means of successive stable counts. For each experimental variable, triplicate cultures were performed and the results were expressed as the mean cpm plus and minus the standard deviation of the three cultures. Background counts, obtained from Hyamine-scintillator blanks were always subtracted. No correction for quenching was made as results (see next section) indicated that the amount of quenching varied little during any one culture.

XIV. Estimation of quenching and counting efficiency.

Quenching was investigated using the external standard channel ratio method (Birks, J.B.). A set of samples containing a known activity and varying amounts of a quenching agent was used to obtain a calibration curve of beta channel counting efficiency (E) versus the external standard channel ratio (B/A) Ex. Std.

Calibration curves obtained with Hyamine hydroxide and two quenching agents, methanol and acetone, gave very similar results. The results presented here are those obtained using acetone, which, because it was a stronger quencher than methanol allowed a wider range of quenching to be calibrated. Scintillation vials were set up to contain varying amounts of Hyamine hydroxide and acetone as shown in table 2.2. Hyamine was used as it was always present in experimental scintillation counting vials. Tritiated toluene (4-(^{3}H) toluene), specific activity 4.71×10^{6} dpm per gm. toluene on the 1st October, 1973, batch 19, Amersham was a gift from Dr. W. Miller, Department of Clinical Surgery. 250 ml. of scintillation fluid was made up to contain 189,000 dpm per 10 ml. (assuming decay of tritium proceeding at 50% per 12.35 years). 10 ml. of scintillation fluid containing the tritiated toluene was then added to each scintillation vial. The vials were kept at 4°C for 1½ hours and allowed to reach a stable temperature in the counter before being counted with the external standard. The results are shown in table 2.2 and the calibration curve in figure 2.1. Within the range of quenching encountered in samples from lymphocyte cultures, the relationship between efficiency and external standard channel ratio can be considered linear. An idea of the extent and variability of quenching can be seen in table 2.3 where data from five representative cultures has been compiled.

Some variations in quenching occurred both within and between cultures. However, since each culture usually served as its own control and variations in quenching were small in relation to variations in isotope incorporation due to other factors, no correction for these small differences was routinely made. However, quenching was routinely monitored by this method and served to delineate vials counting at abnormal efficiency. This occurred, not infrequently when, during the solubilization process the top of the glass tube cracked or was not sealed properly. In these cases the Hyamine solutions were either coloured red and very heavily quenched or in lesser cases the solution was colourless but was nevertheless quite heavily quenched. In such cases a correction was applied or the result was discarded.

Table 2.2 **Results from quench correction experiment in which the efficiency of β channel counting and the external standard channel ratio (B/A. Ext. Std.) was measured in vials containing tritiated-toluene and varying amounts of hyamine and acetone.**

Scintillation vial		Third count cpm	(B/A) Ext. Std.	Efficiency (%)	Fourth count cpm	(B/A) Ext. Std.	Efficiency (%)
Hyamine hydroxide μl.	Acetone μl.						
0	0	81,334	2.3687	43.03	81,301	2.3877	43.01
100	0	70,639	2.3183	37.37	79,425	2.2932	37.26
300	0	59,816	2.1479	31.64	59,650	2.1538	31.56
500	0	52,107	1.9681	27.56	52,004	1.9752	27.51
500	10	50,054	1.9156	26.48	49,969	1.9132	26.44
500	25	42,497	1.7633	22.49	42,461	1.7415	22.47
500	50	35,862	1.6021	18.97	35,821	1.5980	18.95
500	100	26,227	1.3599	13.87	26,199	1.3609	13.86
500	300	12,620	0.9069	6.67	12,498	0.9028	6.61
500	500	6,463	0.6093	3.41	6,447	0.6073	3.41

Table 2.3 The variation in counting efficiency within and between five representative cultures.

Culture	Number of samples	Experimental samples			hyamine-scintillator blank	standard
		B/A External standard channel ratio		Efficiency (%) mean (range)	(B/A) external standard channel ratio	
		Mean ± SD	range			
(K_2)	97	1.9289 ± 0.0316	1.8350 - 2.0020	26.55 (24.4 - 28.2)	1.9833	-
(O_2)	72	2.0937 ± 0.0509	2.0200 - 2.2136	30.3 (28.6 - 33.0)	2.0900	2.4595
(P_2)	87	2.0450 ± 0.0316	1.9910 - 2.1178	29.2 (27.95 - 30.85)	2.1027	2.4649
(Q_2)	73	2.0277 ± 0.0346	1.8964 - 2.1127	28.8 (25.8 - 30.7)	2.0818	2.4489
(R_2)	66	1.9835 ± 0.0223	1.9333 - 2.0415	27.8 (26.6 - 29.1)	2.0273	2.5104

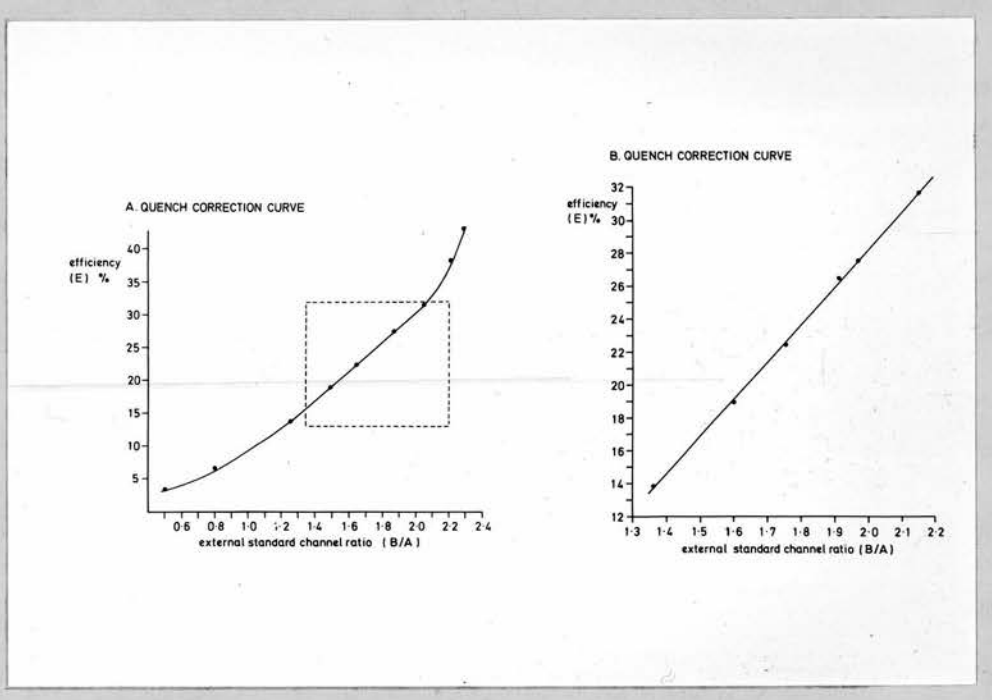


Figure 2.1 (A) Quench correction curve of β counting efficiency and the external standard channel ratio (B/A). Results were obtained using acetone and Hyamine as quenching agents.

(B) Close-up of area shown in figure (A) in which a linear relationship exists between counting efficiency and the external standard channel ratio (B/A).

EXPERIMENTAL DESIGN AND RESULTS

I. Whole blood culture.

Experiments:

(1) A comparison of the whole blood PHA response of a human, a neonatally thymectomized rat and a control rat.

(2) Dose response to PHA.

(3) Effect of pulse time on isotope incorporation.

(4) A comparison of the whole blood PHA response of a group of intact rats and a group of neonatally thymectomized rats.

(5) Quantitation of the proliferative response to PHA using intact rat and thymectomized rat whole blood mixtures.

II. Lymphoid cell culture.

Experiments:

(1) A typical spleen cell culture without added erythrocytes.

(2) A comparison of the ability of different tissue culture media, to support spleen cell cultures.

(3) A comparison of the ability of medium supplemented with either human, rat or FCS to support spleen cell cultures.

(4) Thymus and spleen cell culture, the effect of the addition of erythrocytes.

(5) Quantitation of the spleen cell response to PHA in culture with whole blood from a thymectomized rat.

(6) The effect of HEPES and erythrocytes in cultures of whole blood, spleen and bone marrow cells.

(7) The effect of variations in the number of erythrocytes in spleen and bone marrow cell cultures.

(8) The effect of delayed additions of erythrocytes to cultures of spleen and bone marrow cells

III. Reproducibility of the method.

I. Whole Blood Culture.

Experiment (1) A comparison of the whole blood PHA response of a human, a neonatally thymectomized rat and a control rat.

This culture compared the response of whole blood from an intact rat, an age-matched neonatally thymectomized rat and one human to PHA stimulation, table 2.4.

A 6 - 7 fold difference in the total uptake of isotope was found between the blood of the intact rat and that of the thymectomized rat. If correction was made for lymphocyte numbers, a 2 - fold difference in isotope incorporation was found between the intact and the thymectomized rat. The human blood sample was found to be more responsive than both rat blood samples. Whether this was due to better survival of human lymphoid cells or intrinsic differences in the responsiveness to PHA was not known. Considerable haemolysis had occurred in the rat whole blood cultures by 72 hours, at which time haemolysis was much less evident in human blood cultures. This was a constant finding in cultures of rat and human whole blood.

Table 2.4 Comparison of isotope incorporation in PHA-stimulated and unstimulated cultures of human, intact rat and thymectomized rat whole blood.

Whole Blood	Number of lymphocytes per culture ($x10^{-6}$)	2.5 μl. PHA	0 μl. PHA	SI.*
		cpm., mean ± S.D.		
human	0.2405	155,867 ± 5,637	230	677
intact rat	0.5251	109,054 ± 8,351	244 ± 157	448
Tx rat	0.1676	16,409 ± 3,300	366 ± 193	45

* SI. Stimulation index (ratio of cpm of PHA-stimulated to unstimulated cells).

Experiment (2) Dose response to PHA

A dose response curve was obtained by culturing whole blood from an intact rat and a thymectomized rat with PHA over the range 0 - 12.5 μl. PHA per culture. The results are shown in table 2.5.

The optimal concentration of PHA for the intact rat whole blood was 4.0 μl. per culture, at higher and lower PHA concentrations isotope uptake was reduced. In contrast, the blood from the thymectomized rat showed no optimal dose of PHA, an increased isotope uptake was found with each increase in the concentration of PHA over the range studied. This is shown in figure 2.2. The concentration of PHA that discriminated most between the intact and the thymectomized rat whole blood samples was 1.25 μl. PHA per culture, at which concentration there was a 13 - fold greater total isotope uptake in cultures of intact blood. The blood picture of the two rats is shown in table 2.6 . If the radioactive counts are adjusted for differences in lymphocyte numbers (table 2.7 and figure 2.3) then lymphocytes from the intact rat were found to be more responsive than lymphocytes from the thymectomized rat over the lower concentration range of PHA. However, at the highest concentration of PHA (12.5 μl. per culture), the lymphocytes from the thymectomized rat were found to incorporate more isotope, on a cell to cell basis, than lymphocytes from the intact rat.

Table 2.5 Response of whole blood from an intact rat and a thymectomized rat over a range of PHA concentrations.

PHA µl./culture	Intact rat whole blood		Tx rat whole blood		Ratio intact: Tx whole blood PHA response.
	cpm, mean ± SD	SI	cpm, mean ± SD	SI	
0	81 ± 37	-	158 ± 96	-	0.5
0.625	12,375 ± 2,680	153	1,345 ± 106	9	9.1
1.25	44,007 ± 9,290	543	3,320 ± 716	21	13.3
2.5	92,258 ± 1,317	1139	9,736 ± 416	62	9.5
4.0	108,252 ± 3,307	1336	20,175 ± 1,483	128	5.4
8.0	94,583 ± 15,201	1168	26,111 ± 3,460	165	3.6
12.5	84,161 ± 7,311	1039	40,295 ± 2,750	255	2.1

Table 2.6 Blood picture of the intact and the thymectomized rat used in the PHA dose response experiment.

Whole blood	Total WBC /mm^3.	Total lym. /mm^3.	% lym.	Total poly. /mm^3.	% poly.	Total mono. /mm^3.	% mono.
Intact rat	6,440	5,809	90.2	600	9.3	31	0.5
Tx rat	3,240	2,538	78.3	568	17.5	134	4.1

Table 2.7 Isotope incorporated per 10^6 lymphocytes in cultures of intact and thymectomized rat whole blood over a range of PHA concentrations.

PHA µl./culture	Intact rat whole blood	Tx rat whole blood	Ratio of the PHA response of intact: Tx rat whole blood.
	Isotope incorporation per 10^6 lymphocytes, cpm.		
0	139	623	0.2
0.625	21,303	5,335	4.0
1.25	75,756	13,081	5.8
2.5	158,819	38,360	4.2
4.0	186,352	79,491	2.3
8.0	162,821	102,880	1.6
12,5	144,880	158,766	0.9

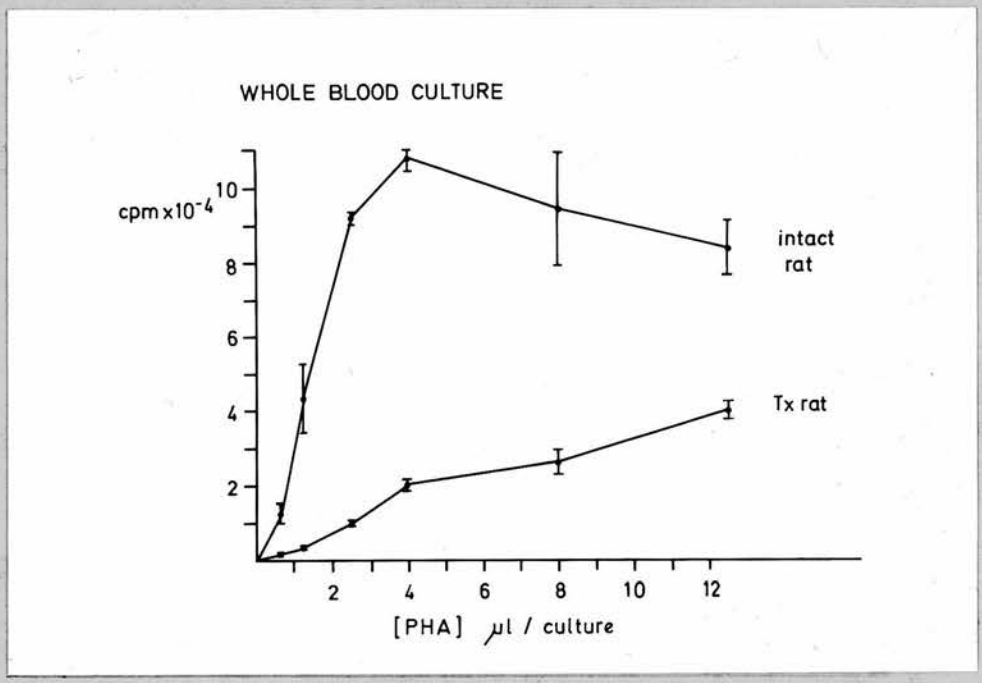


Figure 2.2 Dose response curve for PHA. Whole blood from an intact and a thymectomized rat was cultured in EBM and the response to PHA assessed by measuring tritiated thymidine uptake. The vertical lines represent plus or minus one standard deviation from the mean of triplicate cultures.

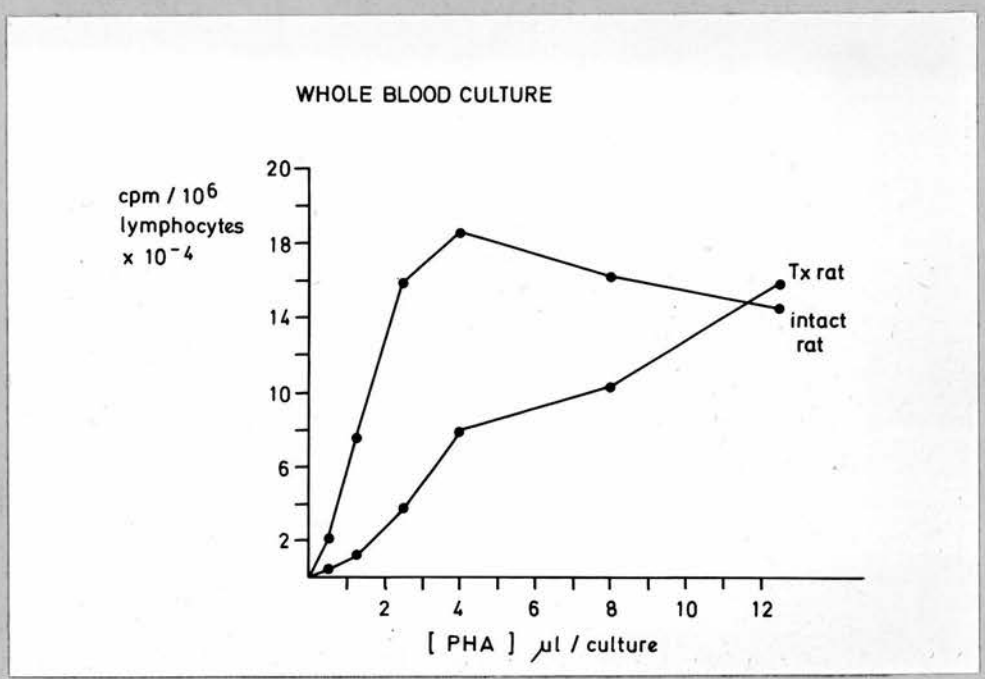


Figure 2.3 Dose-response curve for PHA. Whole blood from an intact and a thymectomized rat was cultured in EBM and the response to PHA assessed by measuring tritiated thymidine uptake. Results are expressed as isotope incorporation per 10^6 lymphocytes.

Experiment (3) Effect of pulse time on isotope incorporation.

Whole blood from an intact rat and a thymectomized rat was cultured in the presence of 4 μl. PHA for 48 hours when 1 μCi. of tritiated thymidine (specific activity 26 Ci/m.mol) was added to each vial. Triplicate cultures from each rat were terminated at 2, 4, 8, 12, 16, 20 and 24 hours after the addition of the isotope. The results of this experiment are shown in table 2.8.

A constant rate of uptake of tritiated thymidine was maintained for 8 hours in the case of intact rat whole blood and for 12 hours for the thymectomized rat whole blood, figure 2.4. After these times isotope uptake tended to level off. In an identically designed experiment the rate of isotope uptake in PHA-stimulated human whole blood cultures was linear for 8 hours then levelled off. These results are not presented in this thesis.

Although better discrimination between intact and thymectomized rat blood was obtained with short pulses (2 - 8 hours), it was decided that the convenience of the 24 hour pulse outweighed the disadvantages. The pros. and cons. of the 24 hour pulse are considered in the discussion.

Table 2.8 The effect of variation in the isotope pulse time on isotope incorporation in PHA-stimulated whole blood cultures. Tritiated thymidine was added to intact and thymectomized rat whole blood after 48 hours in culture and triplicate cultures were terminated following pulse times of 2 to 24 hours.

Pulse time (hours)	Rat whole blood		Ratio response intact: Tx rat whole blood.
	Intact	Tx.	
	cpm., mean ± SD.		
2	29,213 ± 14,551	308 ± 232	95
4	45,080 ± 2,487	349 ± 132	129
8	82,870 ± 17,079	982 ± 257	84
12	82,419 ± 18,930	1,434 ± 419	57
16	110,577 ± 3,245	1,376 ± 464	80
20	106,981 ± 3,765	1,445 ± 323	74
24	103,052 ± 13,131	1,626 ± 195	63

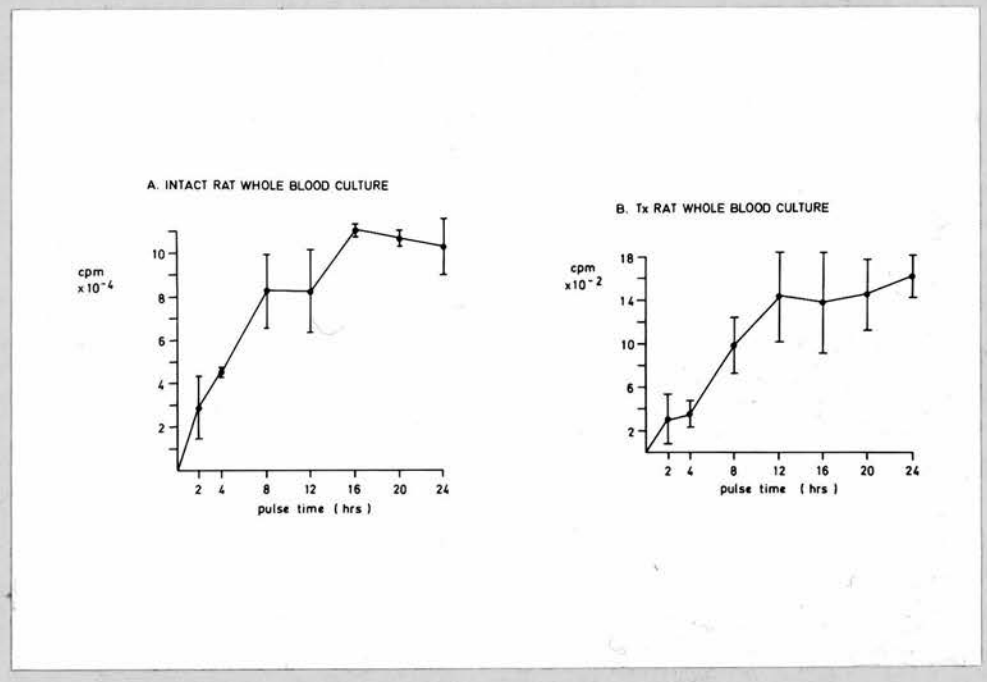


Figure 2.4 Effect of pulse duration on the incorporation of tritiated thymidine in (A) intact, and (B) thymectomized rat whole blood cultures stimulated with 4 μl. PHA. The isotope was added to the cultures after 48 hours and the cultures were terminated 2 to 24 hours later. The vertical lines represent plus and minus one standard deviation from the mean of triplicate cultures.

Experiment (4) A comparison of the whole blood PHA response of a group of intact rats and a group of neonatelly thymectomized rats.

Whole blood was obtained by cardiac puncture from six thymectomized rats aged 8 weeks and five unoperated rats aged 10 weeks. All the rats in this experiment were male. The blood picture of these two groups is shown in table 2.9.

Analysis by the Wilcoxon test suggests that rats from the thymectomized group had significantly fewer white blood cells and specifically, lymphocytes than the control rats.

Whole blood response to 4 μl. PHA is shown in figure 2.5. It can be seen that neonatal thymectomy significantly depressed the PHA response. However, when the radioactive counts are expressed as cpm per 10^6 lymphocytes, figure 2.5, the difference between the means of the two groups is not statistically significant.

Five out of the six thymectomixed rats showed no thymic remnant at autopsy, however, for one rat an autopsy could not be performed. This rat was the one whose PHA response, though not its blood lymphocyte count, was much higher than the other thymectomized rats. It was therefore possible that this rat had been inadequately thymectomized. The relationship between total lymphocyte count and PHA response is shown in figure 2.6. The data was subjected to linear regression analysis and a straight line constructed accordingly. The linear correlation coefficient (r) for the data is 0.872 and this is highly significantly ($2\alpha < 0.001$) different from zero. However, the number of points is small and a better fit to the data is perhaps that represented by the curve.

Table 2.9 Blood picture of group of intact rats and group of thymectomized rats experiment (4).

Rats (number)	Total WBC/mm^3 mean ± S.D.	Total lym/mm^3 mean ± S.D.	lym. %	Total poly/mm^3 mean ± S.D.	poly %	Total mono/mm^3 mean ± S.D.	mono %
Intact (5)	8,032 ± 2,341	6,844 ± 2,107	85.2	1,052 ± 249	13.1	136 ± 35	1.7
Tx (6)	3,000 ± 787	2,316 ± 547	77.2	603 ± 262	20.1	80 ± 44	2.7
Wilcoxon rank test	$p < 0.01$	$p < 0.01$	-	*ND	-	ND	-

* ND - Not done.

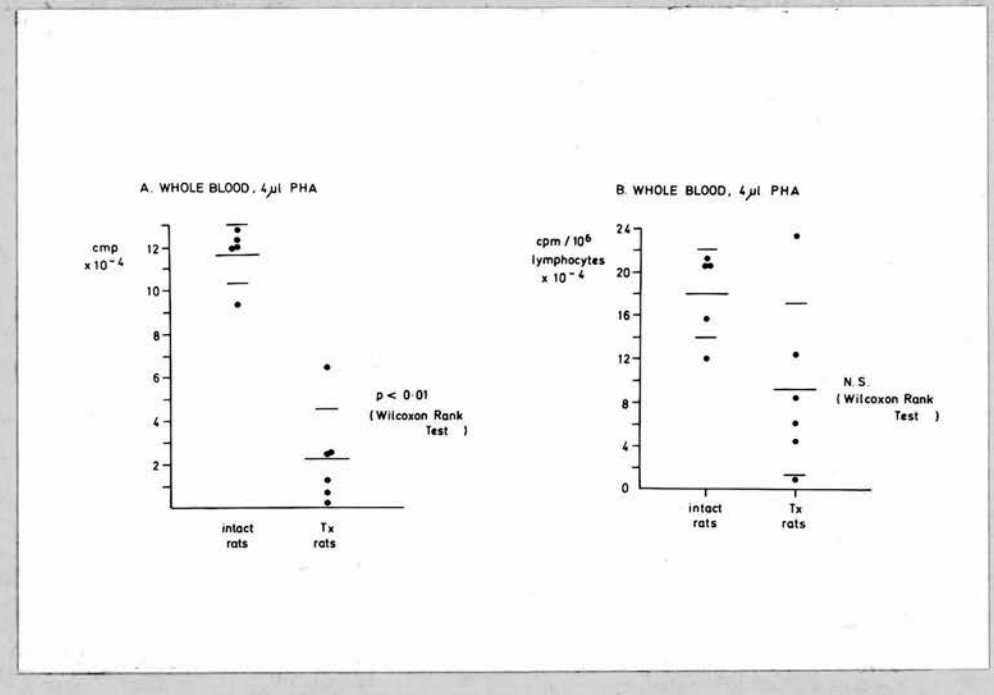


Figure 2.5 A comparison of the whole blood response to 4 μl. PHA of intact rats and neonatally thymectomized rats. (A) Response to PHA expressed as total isotope uptake per culture; means of two groups significantly different. (B) Response to PHA expressed as isotope uptake per 10^6 lymphocytes; means of two groups not significantly different.

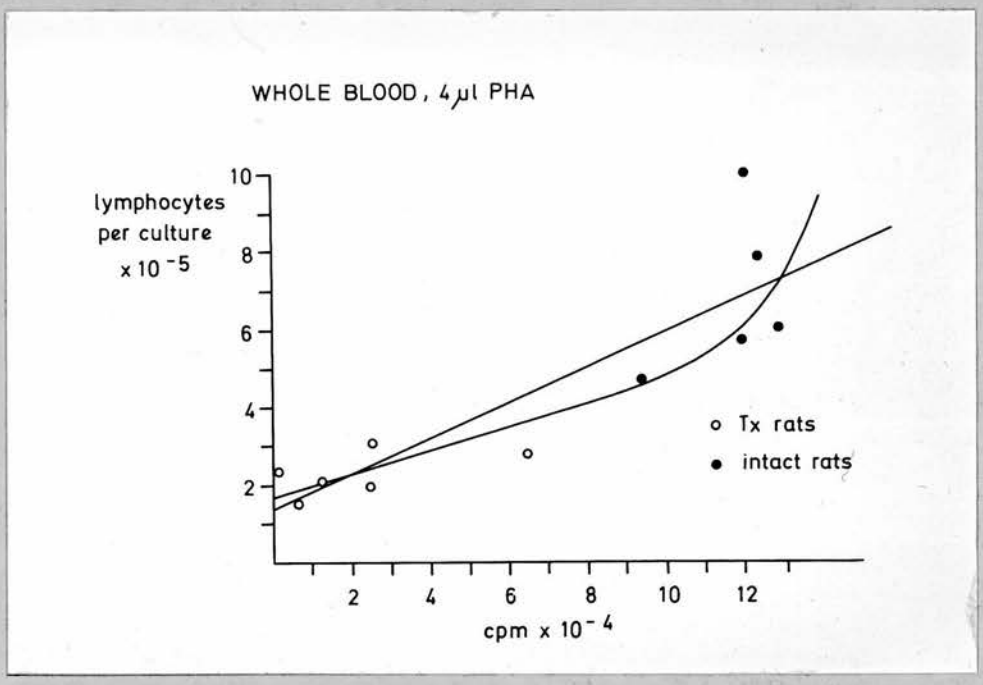


Figure 2.6. **Relationship between the number of lymphocytes per culture and the total isotope incorporation in response to 4 µl. PHA in whole blood cultures of intact and neonatally thymectomized rats. Straight line constructed by correlation of linear regression.**

Experiment (5) Quantitation of the proliferative response to PHA using intact rat and thymectomized rat whole blood mixtures.

An attempt was made to obtain a better understanding of the relationship between isotope uptake and the number of cells participating in the response to PHA.

Whole blood from a thymectomized rat was mixed with blood from an intact rat to obtain the following blood mixtures - 4:0, 3:1, 2:2, 1:3, 0:4. A standard blood culture was then performed in medium 199 containing 10% FCS. The results of this culture are shown in table 2.10.

The relationship between isotope incorporation and blood ratio is shown in figure 2.7 and between isotops incorporation and total lymphocyte count in figure 2.8. It can be seen that for blood mixtures containing 0% to 50% intact rat blood an approximately linear relationship existed between isotope incorporation and total lymphocyte number. For blood mixtures containing 50% to 100% intact rat blood, the linear relationship broke down, although increases in lymphocyte number still led to increased isotope uptake.

Table 2.10 Response to 4 μl. PHA of whole blood mixtures prepared from an intact and thymectomized rat.

Blood mixture	Total lymphocytes per culture ($x10^{-6}$)	cpm, mean ± SD
Tx	0.6817	17,685± 902
3 Tx : 1 INT	0.7909	53,259±3,037
2 Tx : 2 INT	0.9061	80,030±4,856
1 Tx : 3 INT	1.0252	93,423±8,434
INT	1.1527	102,939±15,428

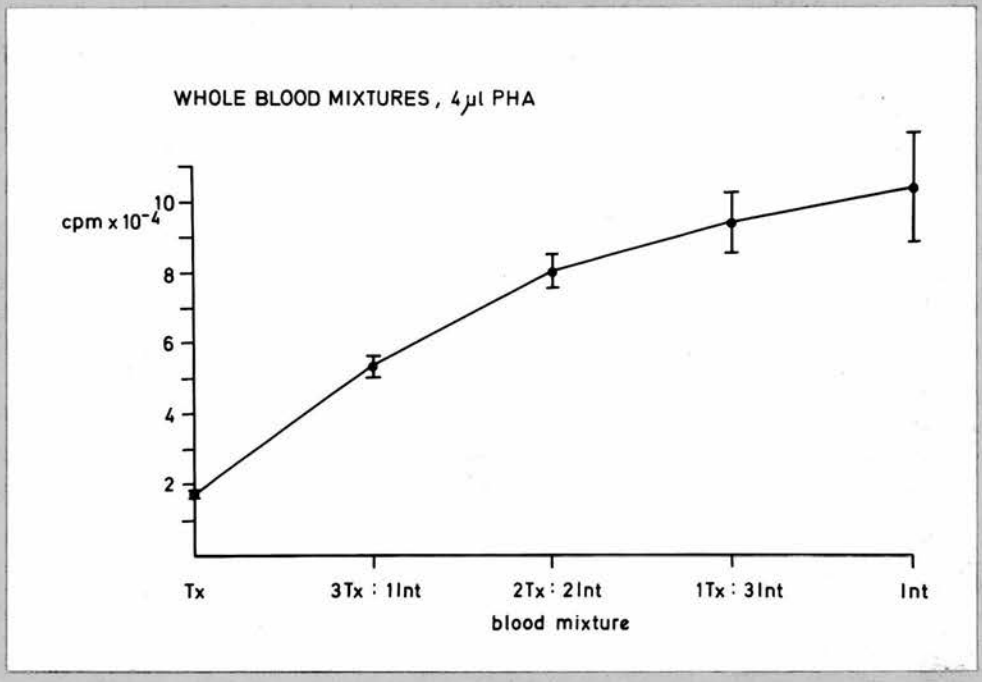


Figure 2.7 Relationship between the proportion of intact and thymectomized rat blood in whole blood mixtures and isotope incorporation in response to 4 µl. PHA. Vertical lines represent plus and minus one standard deviation from the mean of triplicate cultures.

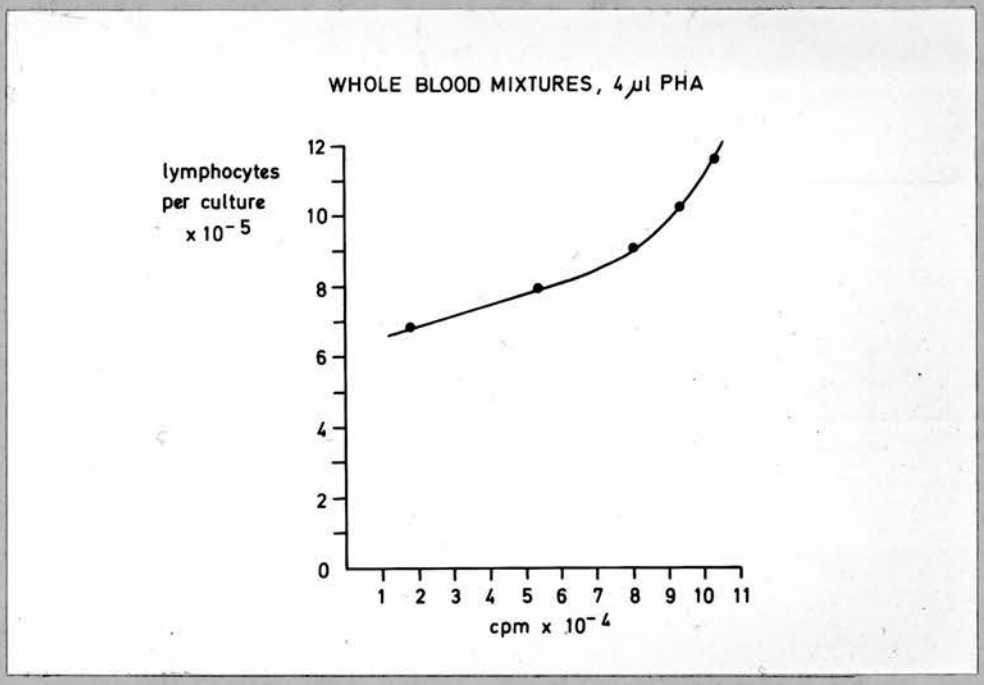


Figure 2.8 **Relationship between the number of lymphocytes in mixtures of intact and thymectomized rat whole blood and the isotope incorporation in response to stimulation by 4 µl. PHA.**

II. Lymphoid Cell Culture.

When the method for whole blood culture was adapted for the culture of lymphocytes separated from rat lymphoid organs, initially, the only change made was that instead of whole blood, 0.1 ml. of separated lymphoid cells was added. This culture method, however, proved inadequate and trypan blue dye-exclusion studies showed that almost all the lymphocytes were dead by 72 hours. An investigation into possible reasons for the failure of lymphoid cells to survive was conducted and eventually a culture method based on the addition of erythrocytes obtained from a thymectomized rat was found satisfactory. The results obtained from this investigation are presented in the following section.

Experiment (1) A typical spleen cell culture without added erythrocytes.

2×10^6 spleen cells from an intact male rat were cultured in EBM in the presence and absence of serum obtained from a thymectomized rat or an intact rat and stored at -20^0C. for one month. The results are presented in table 2.11. It can be seen that in the presence of 10% intact rat serum the optimal concentration of PHA was 2.5 μl. per culture. The presence of serum in the culture medium was found to be necessary for cell survival. Data illustrating this is shown in table 2.12. Isotope uptake was found to be slightly greater in cultures containing 10% serum than in cultures containing 20% serum. When the maximum isotope uptake for spleen cells, almost 4,000 cpm, was compared to that obtained for whole blood from the same rat, in the same culture, 144,000 cpm, it was thought likely that there was a deficiency in the culture system. The ability of different tissue culture media and serum supplements to support spleen cell culture was investigated and the results are presented in the following two experiments.

Table 2.11 Response of 2.0×10^6 intact rat spleen cells in 10% intact rat serum over a range of PHA concentrations.

PHA µl. / culture	cpm mean ± S.D.	SI
0	144 ± 44	-
0.625	2,104 ± 973	15
1.25	2,692 ± 188	19
2.5	3,879 ± 1,410	27
4.0	2,530 ± 839	18
8.0	1,460 ± 285	10
12.5	846 ± 405	6

Table 2.12 Isotope incorporation in PHA-stimulated and unstimulated cultures of 2.0×10^6 intact rat spleen cells in the presence and absence of intact or thymectomized rat serum.

Rat serum		2.5 µl. PHA	0 µl. PHA	SI
%	type	cpm., mean ± S.D.		
0	-	42 ± 28	9 ± 7	5
10	intact	3,879 ± 1,410	144 ± 44	27
	Tx	2,018 ± 463	234 ± 11	9
20	intact	2,884 ± 444	1,137 ± 136	3
	Tx	1,569 ± 119	615 ± 109	3

Experiment (2) A comparison of the ability of different tissue culture media to support spleen cell cultures.

1.3×10^6 spleen cells from an intact rat were cultured in the following media supplemented with 10% FCS: EBM, TC199, and RPMI 1640. The concentration of glutamine was varied to see what effect glutamine has in spleen cell cultures.

The results, presented in table 2.13, show no discernible effect of glutamine. Very low radioactive counts, just above those due to background, were obtained from all cultures.

Table 2.13 Comparison of the effect of different tissue culture media and different concentrations of glutamine on isotope incorporation in PHA-stimulated and unstimulated cultures of 1.3×10^6 intact rat spleen cells.

Medium	l-glutamine (mg/100 ml)	2.5 µl. PHA	0 µl. PHA	SI
		cpm, mean ± S.D.		
RPMI 1640	58.4	28 ± 6	9 ± 4	3
	0	58 ± 33	16 ± 12	4
TC199	58.4	25 ± 19	5 ± 4	5
	0	18 ± 5	5 ± 2	4
EBM	87.6	15 ± 16	4 ± 5	4
	29.2	10 ± 2	49 ± 68	0.2

Experiment (3) A comparison of the ability of medium supplemented with either human, rat or FCS to support spleen cell cultures.

Aliquots of 2.5×10^6 spleen cells, obtained from an intact rat were cultured in medium 199 supplemented with 0, 10% and 20% serum of six different types. The serum was either heat-inactivated or uninactivated. Heat-inactivation was carried out, as described in the methods section, using an air incubator. It was therefore likely that inactivation was incomplete. The human serum (DD) and the rat serum (ADRI) had been prepared on the day before culture. The FCS had been stored at $-20^{\circ}C$. for several months. The results are shown in table 2.14.

The greatest isotope uptake was found in cultures containing FCS. However, as even these cultures did not lead to radioactive counts greater than 2,000 cpm it was thought that the type of serum used was unlikely to be the critical factor in the success or failure of spleen cell cultures. It was shown once again, however, that serum was an essential supplement as cultures containing no serum produced almost no counts.

Heat-inactivation seemed to have no effect on human serum, however it considerably reduced isotope uptake in PHA-stimulated, and to a lesser extent in unstimulated cultures supplemented with ADRI rat serum and FCS. The highest counts in unstimulated cultures were obtained in the presence of autologous (ADRI) serum.

Table 2.14 The effect of different sera on the isotope incorporated by PHA-stimulated and unstimulated cultures containing 2.5 x 10^6 intact rat spleen cells.

Serum			4 µl. PHA	0 µl. PHA	SI
Type	* HI or UNIN	%	cpm, mean ± S.D.		
-	-	0	8±3	19±16	0.4
human	UNIN	10	243±111	31±20	8
		20	1,162±342	28±16	42
	HI	10	261±154	43±35	6
		20	1,197±586	189±72	6
F.C.S.	UNIN	10	1,825±131	219	8
		20	1,956±406	413±84	5
	HI	10	77±27	95±20	0.8
		20	140±22	32±25	4
rat (ADRI)	UNIN	10	1,321±145	549±60	2
		20	1,615±92	612±158	3
	HI	10	529±157	239±78	2
		20	309±44	353±55	0.9

* HI - heat inactivated

UNIN - uninactivated

Experiment (4) Thymus and spleen cell culture, the effect of the addition of erythrocytes.

Since white blood cells survive and respond to PHA in whole blood culture it was decided to investigate whether spleen and thymus cells would respond in a "reconstituted" blood culture.

Whole blood from a thymectomized rat was processed to remove the plasma and most of the white blood cells. Thymus cells, spleen cells and this erythrocyte preparation were then cultured in medium 199 containing 10% FCS. In some cultures, 100 µl. of the erythrocyte preparation was added to vials containing thymus or spleen cells. The results are presented in table 2.15.

The results suggest that in culture by themselves, thymus cells and spleen cells do not survive but in the more favourable environment brought about by co-culture with erythrocytes, significant responses to PHA can be obtained. The low response of the thymus cells, even in the presence of erythrocytes was thought to be due to the naturally poor responsiveness of thymus cells to PHA.

Table 2.15 The effect of erythrocytes on isotope incorporation in PHA-stimulated and unstimulated rat thymus and spleen cells.

Cells		2.5 µl. PHA	0 µl. PHA	SI
Erythrocytes	Lymphoid	cpm., mean ± S.D.		
-	1.2 x 10^6 thymus	12 ± 9	6 ± 3	2
-	1.3 x 10^6 spleen	25 ± 6	9 ± 4	3
+	-	508 ± 111	*ND	-
+	1.2 x 10^6 thymus	1,268 ± 144	ND	-
+	1.3 x 10^6 spleen	14,418 ± 1,458	ND	-

* ND - not done

Experiment (5) Quantitation of the spleen cell response to PHA with whole blood from a thymectomized rat.

Varying numbers of spleen cells from an intact rat were cultured with whole blood obtained from a thymectomized rat. The culture medium was TC199, supplemented with 10% FCS. The response to 4 μl. PHA was assessed using high specific activity tritiated thymidine (26.7 Ci/m.mol.). The results are shown in table 2.16.

The relationship between spleen cell number and isotope incorporation is shown in figure 2.9. The results show that at low numbers of spleen cells the uptake of isotope was proportional to the number of cells per culture. At higher concentrations of spleen cells, the addition of more cells did not produce a corresponding increase in isotope uptake. This levelling off was similar to that seen in the mixed intact-thymectomized rat whole blood cultures, figures 2.7 and 2.8.

Table 2.16 Isotope incorporation in response to stimulation by 4 μl. PHA in cultures of thymectomized rat whole blood to which varying numbers of intact rat spleen cells had been added.

Tx rat whole blood.	Intact rat spleen cells ($\times 10^6$)	cpm mean ± SD.
+	0	15,565 ± 374
+	0.085	27,914 ± 1,397
+	0.18	42,633 ± 2,007
+	0.33	60,812 ± 4,626
+	0.48	66,160 ± 3,690
+	0.63	63,936 ± 3,085

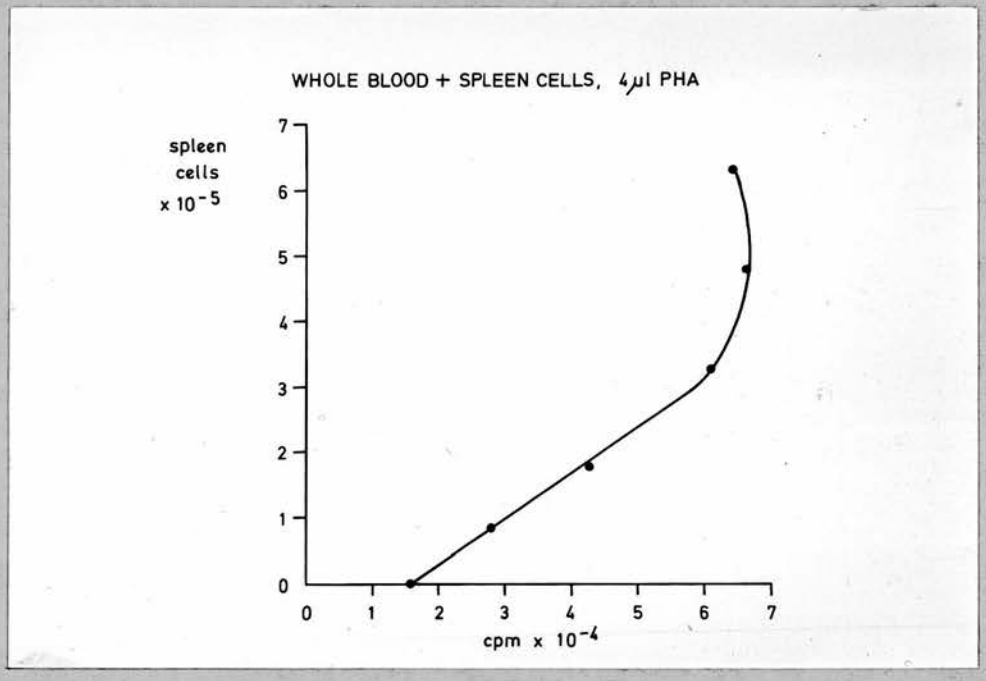


Figure 2.9 **Isotope incorporation in response to stimulation by 4 μl. PHA in cultures of thymectomized rat whole blood to which varying numbers of intact rat spleen cells had been added.**

Experiment (6) The effect of HEPES and erythrocytes in cultures of whole blood, spleen and bone marrow cells.

The success of spleen cell cultures brought about by the addition of erythrocytes was thought to be perhaps due to the buffering ability of the red blood cells. If this was the case it would imply that the bicarbonate-carbon dioxide system, under the conditions used in this study, was inadequate for the buffering of spleen cell cultures. It was decided that the effect of another hydrogen ion buffer should be studied, in combination with bicarbonate. HEPES (4- (2-hydroxy-ethyl) -1-piperazinyl-ethane-2-sulphonic acid) has been found able to maintain a constant pH in cultures of human peripheral blood lymphocytes over the concentration range 10 - 40 mM HEPES, at which concentration it was not toxic and did not itself stimulate lymphocytes (Darzynkiewicz, Z. and Jacobson, B 1971).

The effect of the presence of 10 mM HEPES was studied in cultures of whole blood, spleen and bone marrow cells containing added erythrocytes and in cultures without erythrocytes. All the cultures were done in medium 199 containing 10% FCS. The results of this study are shown in table 2.17.

Once again it was confirmed that the addition of erythrocytes permitted the successful culture of spleen cells and also that of bone marrow cells. The addition of HEPES to the culture medium increased the isotope uptake in whole blood cultures and in spleen cells cultured with erythrocytes. In bone marrow cell cultures, where the response to PHA was small and most of the isotope uptake probably due to active haemat opoietic cells, there was little difference between cultures containing HEPES and cultures without HEPES.

Thus although the presence of HEPES led to greater isotope incorporation in cultures of blood and spleen cells containing erythrocytes, by itself, that is without added erythrocytes, 10 mM HEPES could not sustain spleen or bone marrow cells in culture with PHA.

Table 2.17 The effect of erythrocytes and HEPES on isotope incorporation in PHA-stimulated and unstimulated cultures of intact rat whole blood, spleen and bone marrow cells.

Intact rat cells	Erythrocytes	HEPES (10mM)	2.5 μl. PHA	0 μl. PHA	SI
			cpm., mean ± S.D.		
None	+	+	292 ± 27	266 ± 124	1.1
		-	408 ± 57	315 ± 123	1.3
Whole blood	-	+	12,719 ± 980	192 ± 22	66
		-	7,349 ± 893	249 ± 70	30
Spleen cells. (1.2×10^6)	-	+	40 ± 22	20 ± 2	2
		-	42 ± 12	17 ± 2	2.5
	+	+	25,303 ± 7,206	665 ± 54	38
		-	14,055 ± 547	460 ± 62	31
Bone marrow cells. (2.5×10^6)	-	+	2,376 ± 274	2,735 ± 371	0.9
		-	3,132 ± 265	3,031 ± 609	1.0
	+	+	20,531 ± 1,425	17,076 ± 3,024	1.2
		-	18,688 ± 275	15,759 ± 1,269	1.2

Experiment (7) The effect of variations in the number of erythrocytes in spleen and bone marrow cell cultures.

This experiment and the following investigated how variations in the number of erythrocytes and in the time, after the onset of the culture, that the erythrocytes are added, affect isotope incorporation.

The erythrocytes were prepared from a neonatally thymectomized rat aged four months. Total white cell and red cell counts in the undiluted erythrocyte preparation were as follows: 2,720 WBC per $mm.^3$, 5.5×10^6 RBC per $mm.^3$. The erythrocyte preparation was diluted with medium 199 and the following dilutions made: 1:2, 1:6, and 1:16. Spleen cells and bone marrow cells were cultured in medium 199 containing 125 mg/100 ml. sodium bicarbonate, 10 mM HEPES and 10% FCS. The various erythrocyte preparations were added to the spleen and bone marrow cells at the start of the culture. The results are shown in table 2.18.

If the results from the spleen and bone marrow cell cultures, are compared in terms of "percentage of maximum response", (figure 2.10) it can be seen that the isotope uptake in spleen cells responding to PHA is more sensitive to decreases in the number of erythrocytes than the bone marrow cell response to PHA. The erythrocyte preparation was not cultured in the absence of spleen or bone marrow cells and therefore the magnitude of the contribution of the erythrocyte preparation to the total isotope incorporation was not known. It was thought, however, that the isotope incorporation by the erythrocyte preparation was minimal (less than 500 cpm) in the light of experience with many cultures involving the addition of erythrocyte preparations where the presence of about 272,000 white blood cells was quite normal.

Table 2.18 The effect of variation in the number of erythrocytes on tritiated thymidine incorporation in PHA-stimulated and unstimulated intact rat spleen and bone marrow cells.

Cells (number)	RBC preparation. (dilution)	4 µl. PHA	0 µl. PHA	SI
		cpm., mean ± S.D.		
Intact rat spleen (2.5×10^6)	1/1	77,980 ± 1,460	5,156 ± 213	15
	1/2	22,993 ± 3,691	2,857 ± 85	8
	1/6	10,477 ± 1,267	3,910 ± 361	2.7
	1/16	4,605 ± 552	1,503 ± 70	3.1
	0	5,583 ± 959	570 ± 76	10
Intact rat bone marrow (2.1×10^6)	1/1	18,733 ± 1,322	10,242 ± 17	1.8
	1/2	14,564 ± 1,123	6,886 ± 622	2.2
	1/6	7,764 ± 973	5,533 ± 131	1.4
	1/16	4,980 ± 459	4,022 ± 22	1.2
	0	1,761 ± 322	3,750 ± 10	0.5

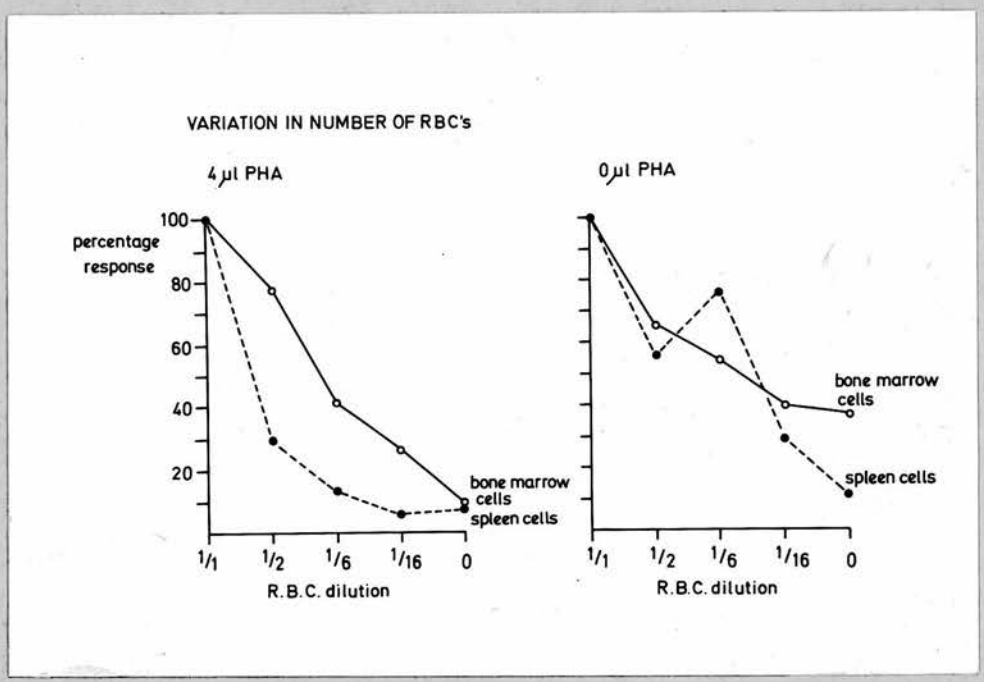


Figure 2.10 The effect of variation in the number of erythrocytes on isotope incorporation in PHA-stimulated and unstimulated cultures of intact rat spleen cells and bone marrow cells.

Experiment (8) <u>The effect of delayed additions of erythrocytes to cultures of spleen and bone marrow cells</u>.

This experiment was undertaken to see if it was possible to delay the addition of erythrocytes to cultures of lymphoid cells without reducing drastically the response to PHA. In certain circumstances it was thought desirable to delay the addition of erythrocytes, for example, in cultures involving prior treatment of the lymphoid cells, as the presence of erythrocytes might obstruct the in vitro modification of the lymphoid cells.

Spleen and bone marrow cells were cultured in medium 199 supplemented with bicarbonate, 10 mM HEPES and 10% FCS. 0.1 ml. of the undiluted erythrocyte preparation (2,720 WBC per mm^3, 5.5×10^6 RBC per mm^3) was added to designated vials at the onset of the culture and at 6, 20 and 47 hours after the initiation of the culture. The culture was terminated after 70 hours. The results are shown in table 2.19.

The results are shown in graphical form in figure 2.11, in terms of the percentage of the maximum response. Any delay in the addition of the erythrocytes to unstimulated cultures resulted in a decreased isotope uptake. In PHA-stimulated cultures, however, when the addition of erythrocytes was delayed for 6 and 20 hours, in the case of spleen cells, and 6 hours for bone marrow cells, a considerable enhancement in the uptake of isotope occurred. It was therefore considered safe to delay the addition of erythrocytes for at least 20 hours in the case of spleen cells and 6 hours for bone marrow cells.

Table 2.19 The effect of delaying the addition of erythrocytes on tritiated thymidine incorporation in PHA-stimulated and unstimulated intact rat spleen and bone marrow cells.

Cells (number)	Delayed addition of RBC (hours)	4 µl. PHA	0 µl. PHA	SI
		cpm., mean ± S.D.		
Intact rat spleen (2.5×10^6)	0	77,980 ± 1,460	5,156 ± 213	15
	6	96,119 ± 6,680	4,057 ± 669	24
	20	108,432 ± 7,102	4,081 ± 1,202	27
	47	8,751 ± 3,117	659 ± 239	13
	not added	5,583 ± 959	570 ± 176	10
Intact rat bone marrow (2.1×10^6)	0	18,733 ± 1,322	10,242 ± 17	1.8
	6	22,409 ± 545	10,045 ± 1,717	2.2
	20	8,741 ± 1,980	7,196 ± 556	1.2
	47	3,616 ± 279	4,129 ± 139	0.9
	not added	1,761 ± 322	3,750 ± 10	0.5

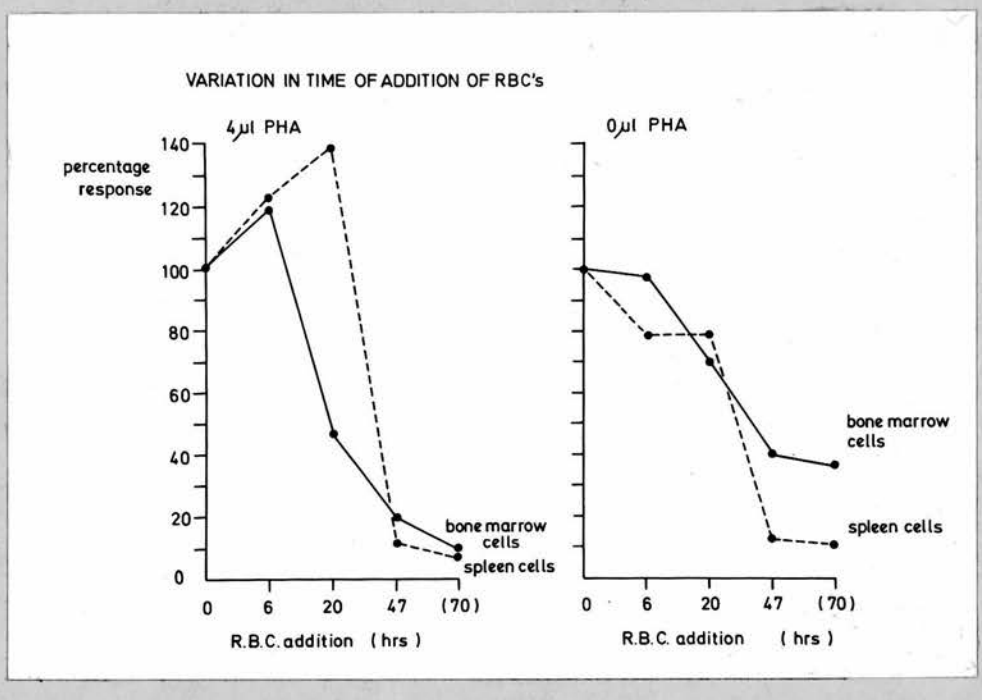


Figure 2.11 The effect of delaying the addition of erythrocytes on isotope incorporation in PHA-stimulated and unstimulated cultures of intact rat spleen cells and bone marrow cells.

III. Reproducibility of the method.

The reproducibility of the methods was analysed by calculating the percentage coefficient of variation between individual counts in triplicate sets of cultures. The coefficient of variation was only computed when the mean of the triplicate counts exceeded 10,000 cpm. This was done to reduce statistical fluctuations due to low mean counts. Several types of culture were analysed: whole blood, washed blood and lymphoid cell culture.

The data depicted in figure 2.12 was obtained from whole blood cultures done on seven different occasions in which a total of 126 samples of blood were cultured in triplicate. In 69% of the triplicate series the percentage coefficient of variation was less than, or equal to, 15%, with the mode between 5 and 10%. Figure 2.12 also shows data from washed blood cultures, where on five occasions, a total of 108 cultures were performed in triplicate. The reproducibility of these results was very similar to those of whole blood. In 70% of the cultures the percentage coefficient of variation was 15% or less and the mode was between 0 and 5%.

The analysis of spleen and thymus cell cultures is shown in figure 2.13 . The data was obtained from two different methods: 'standard' and 'preculture'. The standard method was that in which 100 μl. of the lymphoid cell preparation was added to 1 ml. of medium, followed by the addition of PHA and erythrocytes. The preculture technique involved a 6 - 7 ml. culture for 0. - 24 hours followed by centrifugation and resuspension in the same or a different medium. 1 ml. aliquots were then cultured for 72 hours with PHA and erythrocytes. The results of 35 cultures done by the standard method showed that

the coefficient of variation was 15% or less in 74% (26 out of 35) of the cultures and that the mode was the range 5 to 10%. The preculture method was found to be the most reproducible; in 85% of the samples (47 out of 55) the percentage coefficient of variation was 15% or less and the modal range was between 0 and 5%.

The reproducibility of the whole blood culture method (mean coefficient of variation 12.99%) was similar to the published findings of other workers. Junge reported that in a 100 sets of triplicate cultures the mean variation coefficient of whole blood cultures was 18.2% (Junge, V. et al 1970). Paty and Hughes stated that "with care, groups of five replicates can be cultured, labelled and harvested to give a standard deviation varying between 10 and 20% (Paty, D.W. and Hughes, D. 1972). Biological variation was shown to be the cause of more variation between replicate cultures than errors due to labelling and harvesting (Chalmers, D.G. et al 1967) (Paty, D.W. and Hughes, D 1972).

The greater reproducibility of the methods reported by Schellekens and Eijsvoogel (Schellekens, ETA. and Eijsvoogel 1968) and Penhale (Penhale, W.J. et al 1974), who reported that the coefficient of variation of triplicate cultures was less than 15% in 84% and 93% of all cultures respectively, was only approached by the preculture method. In this method the biological errors were probably reduced by the preculture and the technical errors reduced by the more accurate dispensing of the cells (by 1 ml. aliquots.)

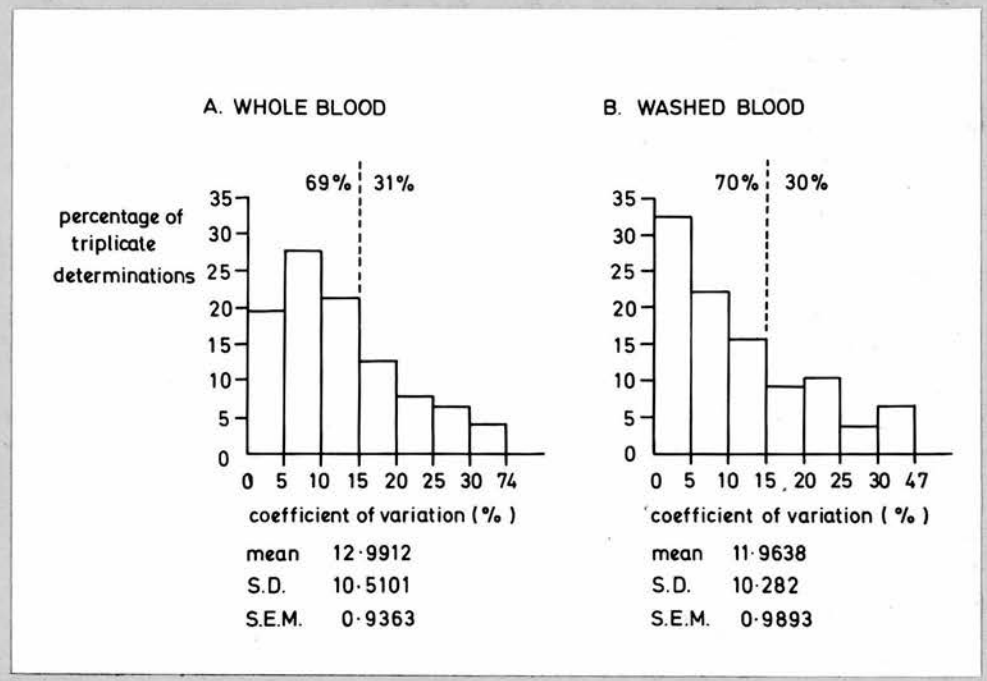


Figure 2.12 Percentage coefficients of variation within triplicate determinations of a series of

(A) whole blood cultures

(B) washed blood cultures.

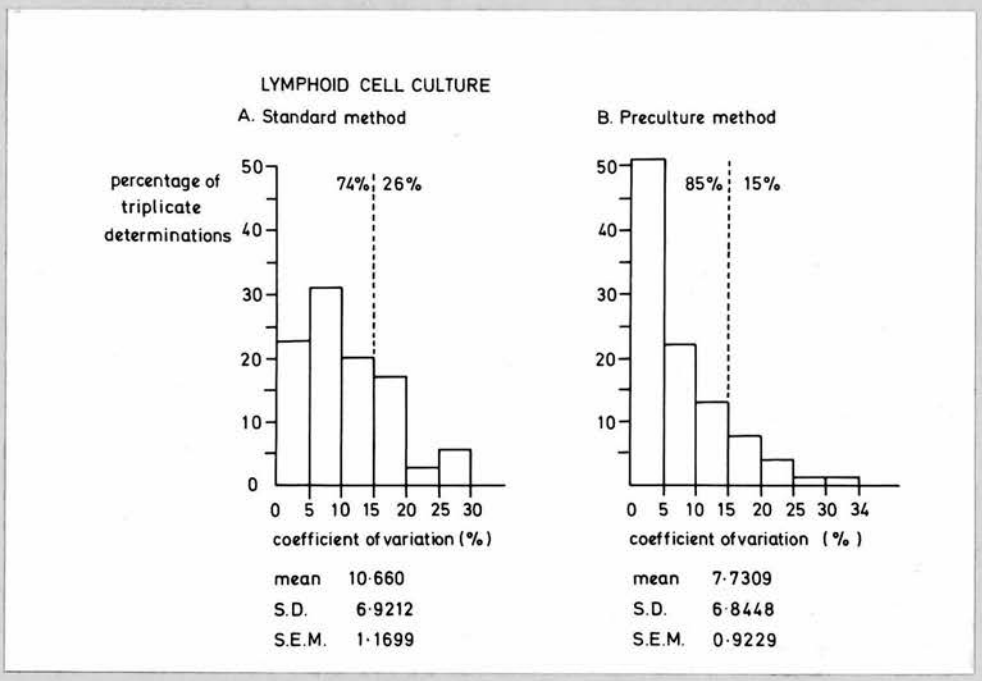


Figure 2.13 Percentage coefficients of variation within triplicate determinations of a series of lymphoid cell cultures using:

(A) the standard culture method

(B) the preculture method.

DISCUSSION

The PHA dose dependence of lymphocyte transformation is well known (Ling, N.L. 1968). The results presented in this thesis were similar to those reported for whole blood culture by other workers (Park, W.H. and Good, R.A. 1972) (Heiniger, H.J. et al 1973). Unpresented results of a PHA dose response curve for human whole blood showed a similar curve, with the optimal dose of PHA lying between 1.25 and 5.0 μl. of PHA per culture. One difference between human and rat blood was that human whole blood responded at PHA concentrations of 0.3125 μl. whilst rat blood was unresponsive at this concentration.

An interesting result of the dose response experiment (figure 2.2) was that no optimal dose of PHA was found for the thymectomized rat over the concentration range studied (0 - 12.5 μl. per culture). At PHA concentrations of 8.0 and 12.5 μl. both supraoptimal doses for the intact rat blood, greater and greater isotope incorporation was found for the thymectomized rat whole blood. Gross differences in the response of the intact and thymectomized rat whole blood can be explained by the absolute and relative peripheral blood T cell deficiency of the thymectomized rat (table 2.6). The different response profiles may also be explained by differences in the number of lymphocytes. Thus, with a higher number of responsive lymphocytes an optimal concentration of PHA is reached at lower PHA doses. Similar findings have been reported by others (Moorehead, J.F. et al 1967). Increased cell densities, up to a certain point, might aid recruitment of cells for transformation through increased cell-cell interactions (Yachnin, S. et al 1972). Greater shifts towards unfavourable culture conditions, such as adverse pH changes and greater utilisation of nutrients in cultures containing more

responding lymphocytes might also be important factors. Another possibility was that serum-PHA interactions were different in cultures of intact and thymectomized rat whole blood. This latter aspect was investigated in detail and the results are described in chapter three.

Similar results to those reported here of the effect of variations in pulse duration on the incorporation of tritiated thymidine by rat whole blood leukocytes have been found for mouse (Heiniger, H.J. et al 1973) and human (Park, W.H. and Good, R.A. 1972) whole blood culture. The decrease from linearity with respect to isotope incorporation with pulse times of 12 hours or more can be attributed to a number of factors. Firstly, the number of cells synthesizing DNA and therefore the total rate of DNA synthesis is unlikely to be constant over 24 hours. Secondly, unless saturating levels of exogenous thymidine are maintained, thus minimising the dilutional effects of endogenous thymidine, the rate of labelled thymidine uptake will not be constant. This was more likely using a precursor of high specific activity and long pulse times. Thirdly, during a long pulse, significant degradation of thymidine to unincorporatable thymine and dihydrothymine may occur.(Marsh, J.C. and Perry, S. 1964). Fourthly, prolonged exposure to β irradiation increases the risk of cell damage or death due to internal irradiation (Drew, R.M. and Painter, R.B. 1962) (Penhale, W.J. et al 1974). Finally, such factors as the accumulation of toxic metabolites or the utilization of essential nutrients, blocking DNA synthesis might be responsible, in part at least, for the levelling off of isotope incorporation during long pulse times.

It has been found that the greatest isotope incorporation in both stimulated and unstimulated cultures was obtained using tritiated thymidine with the highest specific activity (Janossy, G. et al 1973)

(Pauly, J.L. et al 1973) (Penhale, W.J. et al 1974). However, when compounds of very high specific activity were used the sensitivity of the method was reduced i.e. the difference in isotope uptake between responsive and unresponsive lymphocytes was diminished. It was therefore concluded that the specific activity of the DNA precursor should be high enough to produce an easily detected number of radioactive counts and as low as possible to get maximum sensitivity.

The results presented in this chapter were obtained using tritiated thymidine of specific activity 21 to 27 Ci./m.mol. The response to PHA in cultures performed in the second half of this project was assessed using a compound of specific activity 5 Ci./m.mol. This latter compound was not directly compared to the high specific activity tritiated thymidine but was thought, for theoretical reasons, to give more discriminative results. Although better discrimination would have been obtained using short pulse times it was decided for practical reasons, that the 24 hour pulse would be the standard.

The experiment using defined intact and thymectomized rat whole blood mixtures was performed to assess the sensitivity of the culture method. The results showed a near linear relationship over the predominantly thymectomized rat blood range and a gradual levelling off of isotope incorporation as the proportion of intact rat blood increased, figure 2.7. This result, obtained with tritiated thymidine of high specific activity (26.7 Ci./m.mol.) was similar to the findings of others (Janossy, G. et al 1973) and indicated that the sensitivity was greatest for low numbers of responsive cells. This result was therefore consistent with the intention of using the whole blood culture method to measure the reconstitution of the PHA response in thymectomized rats.

A pilot study was conducted in which the whole blood PHA

response of six thymectomized rats was compared to that of five control rats. The results showed statistically significant differences between the groups of intact and thymectomized rats with respect to blood lymphocyte counts and PHA response. The two week age gap between the two groups was unlikely to have made much difference as both groups were physically mature when tested.

In all the blood cultures reported in this thesis, heparin preserved with 0.15% chlorocresol was used. Although heparin is the anticoagulant chosen by most workers, the use of cresol-preserved heparin has been advised against (Waithe, W.I. and Hirschorn, K. 1973). The effect of heparin preserved with chlorocresol, on lymphocyte transformation was not studied.

Several attempts were made to culture lymphoid cells in the absence of added red blood cells. The experiments described in the results section are representative of these attempts. The failure of experiments using different tissue culture media and medium supplements to find conditions permitting successful spleen cell culture, in contrast to the successful method for whole blood culture, stimulated the idea of mixed spleen cell- red cell cultures. Several possibilities were considered to explain the success resulting from the combination of lymphoid cells and erythrocytes. The most likely explanation was based on the buffering ability of erythrocytes (Pentycross, C.R. 1968). Another possibility invoked red cell - PHA - lymphocyte interactions (Tarnvik, A. 1971) (Johnson, R.A. et al 1972) (Yachnin, S. et al 1972).

Commercially available PHA-P when analysed by acrylamide gel electrophoresis was found to contain 17 different protein bands (Allen, L.W. et al 1969). Tarnvik and Yachnin, independently, studied

the two highly purified mitogenic proteins isolated from PHA-P (Tarnvik, A. 1971) (Yachnin, S. et al 1972). One fraction was leucoagglutinating and erythroagglutinating (H-PHAP) whilst the other was leucoagglutinating but only very poorly erythroagglutinating (L-PHAP). Both Yachnin and Tarnvik found that the response to H-PHAP was enhanced by erythrocytes, leucocytes and platelets and that the response to L-PHAP was enhanced by leucocytes and platelets but not by erythrocytes. Complementary results have been found with other haemagglutinating and non-haemagglutinating mitogens (Johnsoh, R.A. et al 1972) (Yachnin, S. 1972 a). The importance of cell membranes in the potentiation of the response to the haemagglutinating mitogen was shown by experiments in which red cell ghosts were found to substitute for intact erythrocytes and particle-free red cell haemolysate preparations were ineffective. A matrix theory for the potentiation of PHAP-induced lymphocyte transformation by cell-cell interactions has been proposed by Yachnin and co-workers (Yachnin, S. et al 1972). This theory took into consideration the subunit structure of PHAP (Weber, T.H. 1969) (Rigas, D.A. and Head, C. 1969) and postulated that the potentiation of the response involved co-precipitation of the red cells, lymphocytes and H-PHAP where a matrix of directionally orientated PHA subunits could be presented to the surface of the lymphocyte thereby allowing simultaneous and more irreversible binding.

The maximum response to PHA was reported by Johnson and co-workers to be at RBC to lymphocyte ratios of 100:1 (Johnson, R.A. et al 1972), and by Yachnin and co-workers to be at ratios of between 5 - 10:1 (Yachnin, S. et al 1972). The results presented in table 2.18 and figure 2.10 show no maxima in the potentiation of the response to PHAP by added erythrocytes. In the case of spleen cells, a red cell to

white cell ratio of approximately 205:1 produced the greatest isotope incorporation and a 102·5:1 ratio gave less than one third of this response. Red cell to white cell ratios of 34:1 and 13:1 led to isotope incorporation no different to that of cultures in which no red cells were added. The isotope incorporation in PHA-stimulated bone marrow cell cultures was not so critically affected by decreases in red cell numbers.

The results shown in table 2.9 and figure 2.11 suggest that PHA activation occurs more efficiently in the absence of red cells (at least for RBC to spleen cell ratios of 205:1 and RBC to bone marrow cell ratios of 244:1) but that the presence of erythrocytes is essential for the expression of this activation. When the addition of erythrocytes was delayed for 20 hours in the case of bone marrow cells and for 47 hours in the case of spleen cells, a considerably reduced PHA response was obtained. However, red cells added at 8 hours and 20 hours to spleen cell cultures and at 8 hours to bone marrow cells allowed a greater isotope incorporation than when erythrocytes were added at the same time as PHA. These results are in partial agreement with those obtained by Tarnvik (Tarnvik, A. 1970). He reported that when erythrocytes were added to putified lymphocytes at various time intervals before the end of the 62 hour incubation period the highest incorporation was found when the erythrocytes were present from the beginning. When the erythrocytes were added 14 hours before the end of the incubation they had no effect on isotope incorporation and erythrocytes added 24 hours after the onset of culture gave a response between these extremes. After adding erythrocytes at the start of the culture, Tarnvik waited 24 hours before adding red cells to another batch of cultures, no data for this interesting period was therefore available for comparison with the results reported here.

The 'erythrocyte-PHAP-lymphocyte' interaction theory as discussed by Yachnin, Tarnvik, and Johnson was considered inadequate to account for the considerable potentiation of isotope uptake brought about by the addition of erythrocytes to lymphoid cell cultures as reported in this thesis. The following results could not be adequately accounted for:-

(1) Very low isotope incorporation in the absence of added erythrocytes.

(2) The response of unstimulated bone marrow and spleen cells was potentiated, although not to the same extent, by the addition of erythrocytes.

(3) Greater PHA activation was obtained in the absence of added erythrocytes providing red cells were subsequently added.

(4) Red cell to lymphocyte ratios required for maximum response far exceeded those reported by other workers

It was therefore thought that the main benefit obtained by coculture with erythrocytes was due to the buffering ability of the erythrocytes. The growth and metabolism of mammalian cells in culture is known to be markedly affected by variations in the pH of ordinary bicarbonate-buffered media (pH 6.9 - 8.0) (Eagle, H. 1971). In spite of its common usage in tissue culture a sodium bicarbonate-CO_2 buffer has two major disadvantages, (1) a CO_2-enriched atmosphere is essential if adequate pH stability is to be achieved (2) the pKa of 6.1 of sodium bicarbonate results in sub-optimal buffering throughout the pH range. During the preparation of cultures, loss of CO_2 from the medium can raise the pH to 8.0. This shift is reversed by the replacement of CO_2 in the atmosphere and as a result of cell metabolism. HEPES has a pKa of 7.31 at 37°C, optimal for buffering at physiological pH and does not require adjustments of atmospheric composition to maintain pH.

Nowell found that the capacity for lymphocyte transformation was not altered between pH 6.9 and 7.7 (Nowell, P.C. 1960). Hughes reported optimal pH conditions for lymphocyte transformation in the range 7.0 to 7.4 with a considerable reduction occurring at pH 7.8 and above (Hughes, D. and Caspary, E.A. 1970). Darzynkiewicz and Jacobson compared bicarbonate and HEPES buffer in lymphocyte cultures, and obtained almost identical results except at higher cell concentrations where HEPES-containing media allowed greater incorporation of tritiated thymidine (Darzynkiewicz, Z. and Jacobson, B. 1971). The optimal response to PHAP was found in the pH range 7.4 to 7.6 in HEPES-buffered cultures.

The result of the experiment in which the effect of 10mM HEPES, in combination with bicarbonate, was studied (table 2.17) showed that the beneficial effect of HEPES was only found in those cultures in which erythrocytes had been added. Without the addition of erythrocytes, medium without HEPES was as effective in supporting lymphocyte transformation as medium containing 10mM HEPES.

At this stage it was decided to use the addition of erythrocytes to lymphoid cell cultures as a means of obtaining a satisfactory response to PHA. The alternative would have been to try higher concentrations of HEPES or to further study the gassing requirement in bicarbonate-buffered cultures.

The PHA response of some of the red cell preparations, obtained from thymectomized rats, is summarized in table 2.20. The response of the red cell preparations to PHA, in cultures without other lymphoid cells, was very low, The total isotope incorporation in combined lymphoid cell - red cell cultures was taken to be mainly due to the lymphoid cells. It was possible, however, that the lymphoid cells potentiated the response of the leukocytes from the red cell preparation.

This aspect was not studied.

The effect of different media on whole blood and lymphoid cell response to PHA was not studied (except in the case of spleen cells cultured without added erythrocytes, table 2.13). Throughout this study EBM and EMEM were used predominantly for whole blood cultures and medium 199 and EMEM for lymphoid cell cultures. All three media proved satisfactory.

The amount of serum or plasma present in the culture medium had a marked effect on the isotope uptake in PHA-stimulated cultures. This is discussed in detail in chapter three. Whole blood cultures were not supplemented with serum and whole blood samples were allowed to retain their autologous plasma (50 - 60% of blood volume). Lymphoid cells were usually cultured in medium supplemented by 5 or 10% serum (usually FCS).

Table 2.20 Isotope incorporation by erythrocyte preparations cultured in the absence of other cells, in the presence of 2.5 µl. or 4.0 µl. PHA.

Number of cultures	Medium	Erythrocyte preparations		
		Total leucocytes per culture		cpm.
		mean ± S.D.	range.	mean ± S.D.
5	TC199 + 10%FCS	192,500 ± 80,006	80,000 - 258,000	244 ± 233
8	EMEM + 10%FCS	106,286 ± 79,695	26,000 - 270,000	47 ± 42

C H A P T E R T H R E E

SERUM STUDIES

INTRODUCTION

The role of serum in lymphocyte transformation.

For reasons which are not entirely clear early attempts to sustain rat lymphocytes in culture during response to PHA met with considerable difficulty . To account for these difficulties Metcalf proposed that the growth of rat lymphocytes was inhibited by a "toxic factor" present in both homologous and autologous rat plasma (Metcalf, W.K. 1965). Thorough washing of the lymphocytes prior to incubation and the use of fresh rat serum appeared to be necessary to ensure the growth of rat lymphocytes (Rieke, W. and Schwartz, M. 1964) (Metcalf, W.K. 1965). The use of serum within one or two hours after its preparation was considered important as serum refrigerated overnight supported decreased and variable growth and serum stored at -20°C. for one to two weeks supported no growth at all. The age, sex or rat strain of the serum donor did not affect the performance of the serum; the only important factor was whether it was fresh or not (Rieke, W. and Schwartz, M. 1964).

Wilson studied the capacity of different sera to support the PHA response of DA strain rat peripheral blood and spleen cells by measuring tritiated-thymidine incorporation (Wilson, D.B. 1967). He found that as 15% solutions, human plasma, calf serum, foetal calf serum and fresh rat serum supported the PHA response, whilst rabbit serum and stored rat serum did not. Meuwissen and co-workers, using Lewis rats, reported that at 20% serum concentrations, fresh heat-inactivated rabbit serum and fresh rat serum supported cell proliferation better than inactivated fresh human serum. Erratic results were obtained with commercial frozen, inactivated rabbit serum (Meuwissen, H.J. et al 1969).

Since these early reports, however, a variety of sera have been

used and the successful culture of rat lymphoid cells has been more or less taken for granted.

The presence of serum or plasma is usually considered essential for the successful culture of lymphocytes with PHA and other mitogens. When the serum was removed by careful washing, and the cells were cultured in medium unsupplemented with serum or plasma, reduced viability and very little reactivity was found to any of the stimulants used (Ling, N.L. 1968) (Hughes, D. and Caspary, E.A. 1970) (Alford, R.H. 1970) (Walker, S. and Lucas, Z.J. 1971) (Pauly, J.L. et al 1973) (Yamamura, M. 1973) (Yachhin, S. and Raymond, J. 1975). Ling recommends that 15 - 20% serum is usually adequate in a good medium such as Eagle's (Ling, N.L. 1968) and most workers have used serum concentrations between 5 - 25%.

Several workers, however, have developed and successfully applied "serum-free" media to lymphocyte culture (Bergman, B. et al 1967) (Coulson, A.S. and Chalmers, D.G. 1967) (Smith, J.L. et al 1967) (Weber, W.T. 1970) (Mattsson, A. and Lindahl-Kiessling, K. 1971) (Vischer, T.L. 1972). These workers used compounds such as dextran, glucose, glucosamine, methyl cellulose and albumin as serum substitutes. It is probable, however, that these cultures were not completely free from serum proteins. For example, it has been reported that repeated washing of cells did not reduce the serum-protein concentration below 100 μg/ml. (Walker, S. and Lucas, Z.J. 1971), presumably due to lysis of the cells during centrifugation. Traces of serum might be expected to be bound to the surface of the lymphocytes. Furthermore, during the culture, serum components, including alpha-globulins could be secreted by the live cells or released from degenerating cells (Furth, R.V. et al 1966) (Smith, J.L. et al 1967) (Mandel, M.A. and Asofsky, R. 1968). Part of the difficulty in analysing the serum requirements of lymphocyte

culture resides in the fact that different workers, have used different cell concentrations, cell populations which were more or less pure with regard to lymphocytes, have differed in the extent to which they washed the cell preparation and have used a variety of criteria for the assessment of the extent of lymphocyte transformation.

The choice of serum for use in lymphocyte culture is of great importance as transformation may be affected by the presence or absence of growth promoting factors, nutrients, antibodies (cytotoxic or enhancing), foreign antigens and specific or non-specific growth inhibitors. Heterologous serum, usually foetal calf serum, has been widely used although there is clear evidence that "spontaneous" transformation can occur in such cultures (Sabesin, S.M. 1965) (Johnson, G.J. and Russell, P. 1965) (Elrod, L.M. and Schrek 1965) (Fikrig, S. et al 1966) (Wilson, D.B. 1966) (Woodliff, H.J. and Onesti, P. 1966). However, Caron observed no spontaneous transformation in cultures of human lymphocytes containing FCS or autologous serum (Caron, G.A. 1967) (Caron, G.A. 1969). He suggested that, at least in some instances, spontaneous transformation has been the result of faulty culture technique or the failure to distinguish blast cells from large macrophages. Pauly found that with human whole blood cultures, spontaneous transformation due to FCS, appeared to be associated with: (1) increasing serum concentration, (2) decreasing blood dilutions, (3) certain batches of FCS, (4) certain blood cell donors whose blood consistently showed a high degree of in vitro responsiveness to the FCS (Pauly, J.L. et al 1973).

PHA has been shown, by gel precipitation techniques, to bind to several components of normal serum. This property was first described by Nakamura and colleagues who found that water-soluble extracts of jack beans and red kidney beans reacted with human and animal serum proteins (Nakamura, S. et al 1960). In 1962 Beckman

demonstrated a reaction between PHA and a serum alpha-globulin fraction and suggested that the mitogenic property of PHA might be due to reaction with a serum factor which normally inhibits lymphocyte transformation (Beckman, L. 1962). This hypothesis has recently received support from the demonstration of specific soluble factors inhibiting lymphocyte proliferation (Houck, J.C. et al 1971) (Kiger, N. 1971) (Houck, J.C. et al 1973). Further support comes from the demonstration that the quantity of PHA which must be added to cultures to achieve a given degree of transformation is directly proportional to the serum concentration, up to a certain concentration of serum (Forsdyke, D.R. 1967) (Walker, S. and Lucas, Z.J. 1971). However, the activation of lymphocytes by PHA in "serum-free" media and furthermore their non-activation in the absence of PHA is a powerful argument against such a mechanism. Morse showed that the main components of normal human serum involved in the precipitation with PHA were macroglobulins, lipoproteins and glycoproteins. Concanavalin A (ConA) was found to precipitate with the same proteins as PHA. PHA-P was able to form a precipitate with serum from humans, monkeys, rabbits, guinea-pigs, rats, calves and ducks. The ability to precipitate with PHA was retained whilst heating normal serum to 56°C but was lost at 85°C (Morse, J.H. 1968).

It is the experience of several workers that the extent of lymphocyte transformation is dependent upon the relative concentrations of serum and PHA (Forsdyke, D.R. 1967) (Alford, R.H. 1970) (Walker, S. and Lucas, Z.J. 1971) (Steinman, H.G. et al 1972) (Forsdyke, D.R. 1973). These workers showad that, at a particular lymphocyte concentration, a stoichiometric relationship exists between the serum protein concentration and the amount of PHA necessary for maximum transformation

at that serum concentration. This relationship holds good in the serum concentration range 3 - 20%. At serum levels greater than 30% a reduced level of transformation occurs regardless of the amount of PHA present (Walker, S. and Lucas, Z.J. 1971). An explanation of this interdependency is that PHA and serum interact with one another to modify the amount of PHA available for the stimulatory process. Such a concept was proposed by Steinman and co-workers (Steinman, H.G. et al 1972) who further showed that the PHA response of mouse spleen cells depended upon the concentration of serum, PHA and lymphocytes. Serum was considered to exert a buffering effect which was expressed as a suppression of the activity of very low concentrations of PHA and as a tolerance of levels of PHA which otherwise would have been inhibitory. Support for this concept came from cultures done in "serum-free" media containing either serum albumin, gelatin or methyl cellulose. Such media supported transformation to a moderate degree and it was found that the cells were very sensitive to the toxic effect of PHA in concentrations above a sharply defined maximum (Steinman, H.G. et al 1972). PHA has been shown not to precipitate with serum albumin (Morse, J.H. 1968) (Osunkova, B.O. and Williams, A.I.O. 1971).

A direct effect of serum in the binding of mitogen to lymphocytes was shown by Möller and co-workers (Möller, G. et al 1973). They found that optimal T cell proliferation occurred when approximately 3×10^6 molecules of ConA were bound per cell, which corresponded to 3 - 10% of the available receptors. If the experiments were performed in the presence of serum, there was a considerable reduction in the binding of the labelled ConA molecules. For example, in 10% FCS, much fewer molecules were taken up at all ConA concentrations.

Another explanation for the effect of serum on lymphocyte

transformation is that the serum factor(s) might modify transformation by binding to either the same site as PHA or a separate site which when filled interferes with the activation process. One of the main difficulties in research into these factors is that of distinguishing between an effect of serum on PHA activation and the subsequent effect on the cellular changes following activation. It has been proposed that whole serum contains two activities (Walker, S. and Lucas, Z.J. 1971). One factor is required, in small amounts, for net RNA synthesis and thymidine incorporation in transforming lymphocytes. It is not known to what extent serum sutstitutes can function in place of this factor. The second factor is a species of molecular weight greater than 10,000, residing in the α-globulin region, and inhibiting lymphocyte transformation. The identity of both factors is not known.

An alpha-glycoprotein present in the serum or plasma of some species has been implicated in the regulation of immune responses. Kamrin in 1958 was the first to describe the immunosuppressive properties of a crude serum alpha-globulin fraction (Kamrin, B.B. 1958). He found that such a fraction obtained from rat serum prolonged allogeneic union (Kamrin, B.B. 1958) and skin graft survival (Kamrin, B.B. 1959) in rats. These findings were extended by Mowbray who further purified the active protein in ox blood by DEAE chromatography (Mowbray, J.F. 1963) This α_2-glycoprotein (fraction C) was able to prolong skin allograft survival in rats (Mowbray, J.F. 1963 a) and suppress haemagglutinin formation in rabbits (Mowbray, J.F. 1963 c). The glycoprotein was only immunosuppressive if given early, a short delay in its administration after the antigenic stimulus led to no immunosuppression. Further studies showed that the immunosuppressive activity was

associated with, and perhaps due to, ribonuclease activity (Mowbray, J.F. 1963 b) (Mowbray, J.F. 1967). Fraction C glycopeptide was also shown to have an inhibitory effect in vitro on the human lymphocyte response to PHA, PPD and allogeneic cells (Milton, J.D. 1971), even when added 24 hours or more after the start of culture.

Mannick and co-workers in 1967 isolated a human α-globulin fraction from normal human plasma using methods similar to those of Mowbray and showed that this fraction prolonged skin allograft survival in rats (Mannick, J.A. and Schmid, K. 1967). This fraction, called Immunoregulatory alpha globulin (IRA), suppressed antigen and PHA induced lymphocyte transformation (Cooperband, S.R. et al 1968 a) (Cooperband, S.R. et al 1969) (Cooperband, S.R. et al 1972) as well as the production of macrophage migration inhibitory factor (MIF) by immune lymphocytes (Cooperband, S.R. et al 1969). IRA suppressed primary and secondary immune responses as measured by the haemolytic plaque assay in mice (Glasgow, A.H. et al 1971) and was also found to impair resistance to tumour growth in mice (Glasgow, A.H. and Mannick, J.A. 1972).

The mechanism of the suppression of PHA transformation by IRA was thought to involve a non-competetive interaction between PHA and IRA for two receptors. Activation of the IRA receptor would inhibit the metabolic events following PHA receptor activation (Cooperband, S.R. et al 1972). It was, however, found that PHA formed a precipitate with IRA in agar diffusion studies. However, incubation of PHA with IRA until no more precipitate formed, and then removal of this precipitate by centrifugation, resulted in no loss of the mitogenic activity of PHA as shown by serial dilutions (Cooperband, S.R. et al 1972). Furthermore,

Yachnin found that an alpha-globulin fraction, provided by Cooperband, suppressed the PHA response of human lymphocytes to both the highly purified mitogens (H-PHAP and L-PHAP), isolated from crude PHA. Only one of the mitogen preparations, H-PHAP, however, formed a precipitate with human serum or the alpha-globulin preparation (Yachnin, S. 1972 b). These results are not in accord with the idea that suppression of PHA response by alpha-globulins is due to precipitation of PHA by the alpha-globulin.

Recent studies by the "IRA group" have resulted in the isolation of a peptide of molecular weight 4,000 to 6,000 obtained from Cohn fraction IV of normal human plasma by ethanol and ammonium sulphate precipitation. (Occhino, J.C. et al 1973). This peptide had the immunosuppressive properties of IRA and was thought to be non-covalently bound to one or more proteins having the electrophoretic mobilities of alpha-globulins.

The existence of an immunosuppressive alpha-globulin in serum has been confirmed in several other laboratories. (Riggio, R.R. et al 1969) (Ashikawa, K. et al 1971) (Glaser, M. et al 1972) (Nelken, D. 1973) (Chase, P.S. 1972) (Veit, B. and Michael, J.G. 1973).

In 1963 Mowbray reported a study concerned with the demonstration of the presence of immunosuppressive globulins in the tissues (Mowbray, J.F. 1963 b). He found that the thymus contained detectable quantities of an immunosuppressive alpha-globulin whilst rat liver, spleen, pancreas and muscle did not. Carpenter extended Mowbray's studies and prepared an alpha-globulin fraction from bovine thymus (Carpenter, C.B. et al 1971 a). This fraction was assayed in vitro by its ability to inhibit DNA synthesis in PHA-stimulated lymphocytes and in vivo by its ability to suppress the haemagglutinin response to sheep erythrocytes. This globulin fraction was shown to have similar properties to those of the

alpha-globulin fractions isolated from plasma. The thymus globulin, however, although it blocked DNA synthesis, had little effect on RNA and protein synthesis and also blast transformation was not prevented (Carpenter, C.B. et al 1971 b). It was concluded that this globulin exerted its modulating effect on immune responses by preventing DNA synthesis in the stimulated cell and not by blocking the early recognition or response to antigen.

Common to most of the in vitro studies on alpha-globulin fractions is the finding that the preparations were not directly toxic to lymphocytes. This has been shown by trypan-blue dye exclusion studies (Cooperband, S.R. et al 1968 a) (Carpenter, C.B. et al 1971 b) (Veit, B.C. and Michael, J.G. 1972) (Glaser, M. et al 1972) (Nelken, D. 1973) (Nelson, D.S. and Shneider, C.N. 1974). The α-globulin fraction described by Mowbray has been shown to have ribonuclease activity (Mowbray, J.F. 1963 b) (Mowbray, J.F. 1967); other alpha-globulin fractions have been ascribed low or no ribonuclease activity (Cooperband, S.R. et al 1969) (Chase, P.S. 1972) (Veit, B. and Michael, J.G. 1973).

In addition to those constituents of normal serum that effect the lymphocyte response to PHA, there has been active interest, over the past few years, in correlating the diminished PHA response occurring in individuals with a variety of physiological and pathological conditions to inhibitory factors in the serum. It is not known whether the postulated serum inhibitors are due to increased amounts of naturally occurring immunosuppressive compounds or due to the presence of abnormal compounds. Another possibility is that the serum might be deficient in compounds that facilitate transformation or its measurement. For example, human serum in pregnancy has been shown to contain a factor or factors depressing the PHA response (Leikin, S.

1972) (St. Hill, C.A. et al 1973) (Von Schoultz, B. et al 1973) (Hsu, C.C.S. 1976). Many reports have claimed that the poor immunological reactivity of patients with malignancy might be due to an immunosuppressive factor present in the serum (Trubowitz, S. et al 1966) (Silk, M. 1967) (Brooks, W.H. et al 1972) (Gatti, R.A. et al 1970) (Sucio-Foca, N. et al 1973) (Catalona, W.J. et al 1973). Other workers have not been able to demonstrate a serum inhibitor in patients with malignancy (Golob, E.K. et al 1969) (Thomas, J.W. et al 1971) (Al-Sarraf, M. et al 1971) (Whitehead, R.H. et al 1974) (Blomgren, H. et al 1975) (Drewitt, D. et al 1975 unpublished findings).

In some cases, a positive correlation between serum alpha-globulins and serum inhibition of PHA response has been found (Ashikawa, K. et al 1971) (Hsu, C.C.S. and LoGerfo, P. 1972); in other instances no such correlation was found (Gatti, R.A. 1971).

Inhibitory factors have also been found in the serum in a variety of other conditions (Hsu, C.C.S. 1976), including; secondary syphilis (Levene, G.M. et al 1969), ataxia telangiectasia (McFarlin, D.E. and Oppenheim, J.J. 1969), advanced alcoholic cirrhosis (Hsu, C.C.S. and Leevy, C.M. 1971), idiopathic steatorrhoea (Winter, G.C.B. et al 1967) and renal failure (Newberry, W.M. and Sanford, J.P. 1971).

EXPERIMENTAL AIMS.

The main aim of the experiments described in this chapter was to investigate serum and plasma from intact and thymectomized rats for possible differences in their ability to support lymphocyte transformation in response to PHA. There were two ways (at least) in which such differences might occur, firstly in the amount of immunosuppressive factors and, secondly in the amount of thymic-dependent humoral factors

which might increase tritiated-thymidine uptake in lymphocytes from normal or immune-deprived rats responding to PHA.

The demonstration of large amounts of immunosuppressive alpha-globulins in bovine thymus glands (Cappenter, C.B. et al 1971 a) which were similar to substances in the serum, although not proving that the thymus was the origin of such substances, suggested the idea of comparing the immunosuppressive potential of serum from intact and thymectomized rats. Jankovic and co-workers found no significant difference, with respect to any of the major plasma proteins (albumin, α, β, γ) between thymectomized and sham-thymectomized rats (Jankovic, B.D. 1962). Measurement of levels of plasma proteins, however, would not reveal functional differences. Nelson investigated a factor, present in normal mouse serum, which depressed DNA, RNA and protein synthesis in PHA-stimulated mouse spleen cells (Nelson, D.S. and Schneider, C.N. 1974). These activities, which were not affected by heating the serum at 56°C. for 20 minutes, were also present in the serum of nude mice (Nelson, D.S. 1972), which made their origin from the thymus or T lymphocytes most unlikely.

Injections of whole serum from intact mice into neonatally thymectomized mice has been reported as ineffective in restoring immunocompetence (Law, L. et al 1964 b) (East, J. and Parrott, D.M.V. 1964). A thymus-dependent factor, has however, been isolated from serum by J-F. Bach and co-workers and thymusin has been demonstrated in the serum using radioimmunoassay (Schulof, R.S. 1972) (Schulof, R.S. et al 1973). These factors are discussed more fully in chapter one. In addition to these factors, known to be thymus-dependent, a factor was found in certain batches of foetal calf serum to restore the normally depressed response of spleen cells from thymectomized mice to respond to sheep erythrocytes in the haemolytic plaque assay (Byrd, W.

1971). The same serum factor did not, however, restore the ability of spleen cells from a thymectomized mouse to respond to PHA. Wilson and co-workers found that the response of thymectomized BN rat lymphocytes, in a mixed lymphocyte culture with normal BN/DA rat lymphocytes was strikingly less than that when BN lymphocytes from an unoperated rat were cultured with normal BN/DA lymphocytes (Wilson, D.B. et al 1967). Furthermore, in cultures containing 15% BN serum it made no difference to the proliferation of either intact or thymectomized BN cells whether the serum donor was an unoperated rat or a thymectomized rat. Carpenter reported the occasional augmentation of PHA response with low concentrations of some alpha-globulin preparations (Carpenter, C.B. et al 1971 a). He could not explain these findings. Similar findings were reported for human IRA: this activity sometimes persisted on storage for several months (Cooperband, S.R. et al 1969).

METHODS

The culture methods used in the experiments described in this chapter were essentially those described in chapter two. When different techniques were used, these are described in the section on experimental design and results.

Tritiated-thymidine of low specific activity (5 Ci./m.mol.) was used exclusively to assess the response to PHA in all the experiments described in this chapter.

EXPERIMENTAL DESIGN AND RESULTS.

Experiments:

(1) The effect of culture with homologous and heterologous sera on the incorporation of tritiated thymidine in cultures of washed rat blood and 4 μl. PHA.

(2) An estimation of the variation in the ability of different samples of normal rat serum to support lymphocyte transformation in response to PHA.

(3) Pooled rat serum studies.

(3a) Comparison of the ability of pooled intact rat serum and pooled thymectomized rat serum to supppport PHA transformation in cultures of intact and thymectomized rat spleen cells.

(3b) Comparison of the ability of pooled intact rat serum and pooled thymectomized rat serum to support PHA transformation in cultures of washed intact rat blood cells and washed thymectomized rat blood cells.

(3c) Comparison of the ability of heat-inactivated pooled intact rat serum and pooled thymectomized rat serum to support PHA transformation in cultures of washed intact rat blood cells and washed thymectomized rat blood cells.

(4) The effect of fresh, intact and thymectomized rat plasma on tritiated thymidine incorporation in PHA-stimulated and unstimulated thymectomized rat spleen cell cultures.

Experiment (1) <u>The effect of culture with homologous and heterologous sera on the incorporation of tritiated-thymidine in cultures of washed rat blood and 4 μl. PHA.</u>

This experiment was carried out to obtain a profile of the effect of different sera on the response of washed intact rat blood to 4 μl. PHA. 10^6 washed rat blood leukocytes were cultured in EBM containing 0 - 25% serum of the following types: human, FCS, rabbit and rat. The human serum (DD) had been stored for five days and the FCS for four months at -20°C before use. The rat serum (ADRI) was obtained by mixing equal volumes of serum from five intact rats. The rats had been bled when 5 - 7 months old and the serum stored at -20°C for about 2 months. The rabbit serum had been heat-inactivated and stored at -20°C for four months.

Medium, serum 4 μl. PHA and the washed blood cells were added to the culture vials and a standard blood culture performed. The results are shown in table 3.1 and figure 3.1.

The maximum response to PHA was found in cultures of 1% human serum. All serum concentrations greater than 1% were found to inhibit DNA synthesis in PHA-stimulated blood cultures. The high isotope incorporation in cultures with no added serum was typical of rat washed blood cultures and spleen cell-erythrocyte cultures. This contrasted with the finding that lymphoid cells, cultured in the absence of erythrocytes, incorporated no tritiated-thymidine in the absence of serum. The inhibition profile of the rabbit serum was different from that of the other three sera. This was possibly because the rabbit serum was heat-inactivated. In a previous culture (results not presented) 10% uninactivated rabbit serum was found to be more inhibitory than 10% FCS or rat serum.

Table 3.1 Response of 10^6 intact rat blood cells to 4 µl. PHA in EBM supplemented with various concentrations of heterologous and homologous serum.

Serum %	Serum			
	Human	FCS	Normal rat	Rabbit *(HI)
	c.p.m., mean ± S.D.			
0	199,509 ± 277			
0.2	222,043 ± 10,847	167,377 ± 7,109	196,767 ± 16,074	202,368 ± 26,610
1	236,257 ± 11,294	144,532 ± 2,383	206,168 ± 6,737	193,197 ± 12,175
5	158,552 ± 18,807	66,150 ± 5,633	121,284 ± 7,252	181,865 ± 12,546
10	86,378 ± 2,287	39,446 ± 637	59,977 ± 11,050	174,517 ± 7,599
25	19,443 ± 5,278	17,264 ± 1,317	18,695 ± 1,145	152,741 ± 13,144

* HI - heat-inactivated.

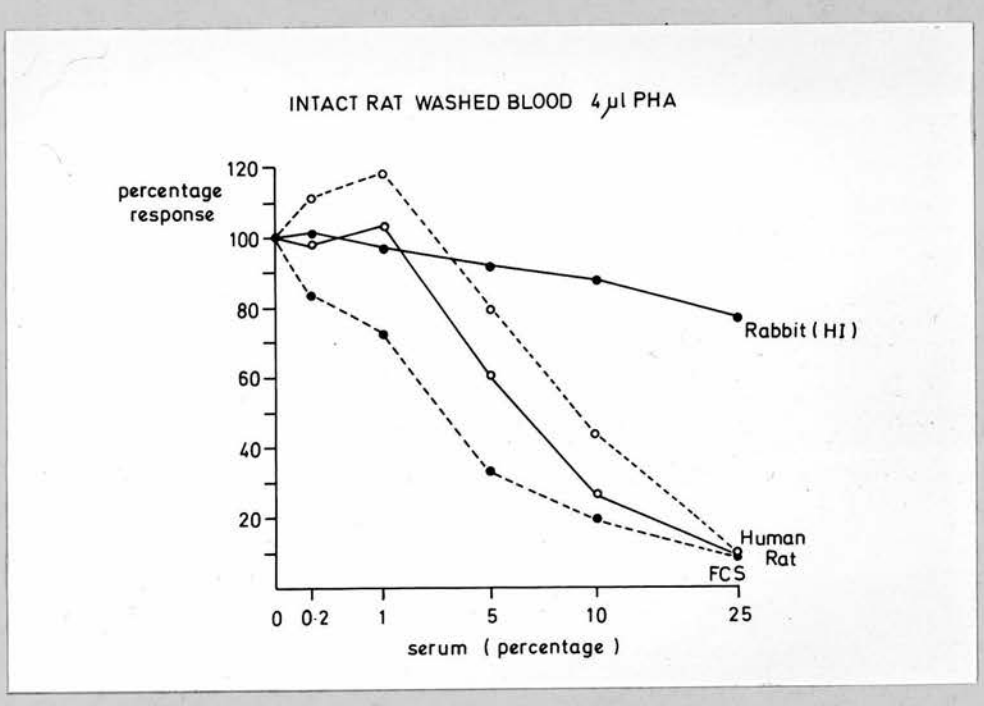


Figure 3.1 Response of 10^6 washed intact rat blood cells to stimulation by 4 µl. PHA in EBM supplemented with either rabbit (heat-inactivated), human, rat or foetal calf serum. Results expressed as a percentage of the isotope incorporated in cultures without serum.

Experiment (2) An estimation of the variation in the ability of different samples of normal rat serum to support lymphocyte transformation in response to PHA.

Serum samples, obtained from age-matched intact male rats were stored for between two and four months at -20°C before use. Eleven serum samples were compared in a single culture using EBM and washed blood cells from an intact rat. The isotope incorporation in response to 4 μl. PHA in 10% serum is shown in figure 3.2, compared to the isotope incorporated in cultures without serum. The striking feature of the results is the variation in isotope incorporation - an almost 7 fold difference between the highest and lowest response. This was not expected as the donors of the serum were genetically identical and age-matched. Isotope incorporation bore no obvious relationship to the length of time the serum had been stored.

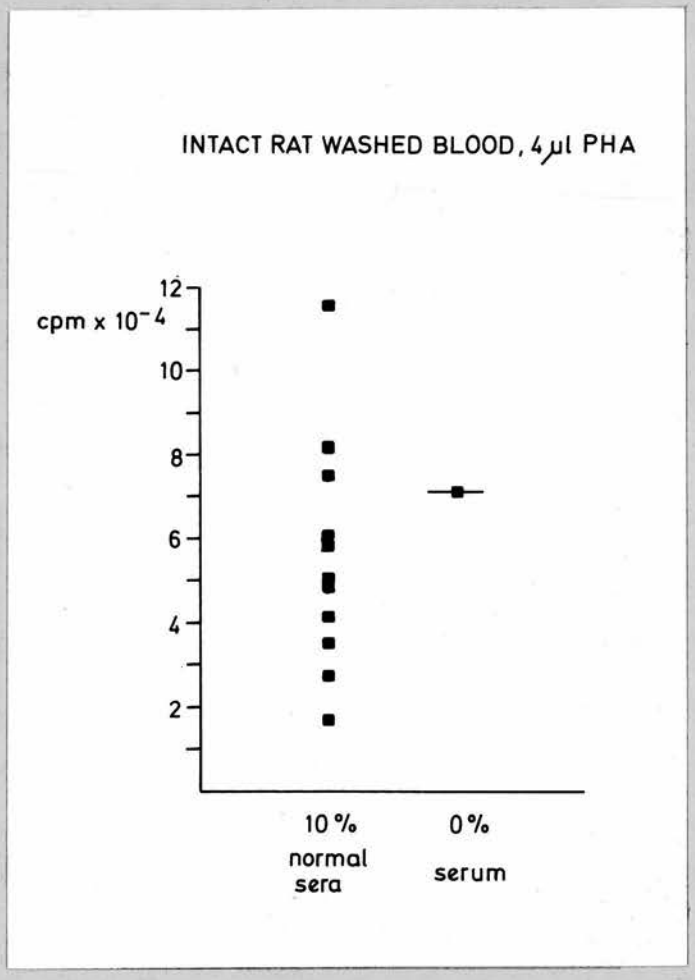


Figure 3.2 A comparison of the response of washed intact rat blood cells to 4 µl. PHA in the presence of eleven samples of serum from intact rats to that of cells cultured in the absence of serum.

Experiment (3) Pooled rat serum studies.

Preparation of intact rat and thymectomized rat serum pools.

As the reasons for the wide range of DNA synthesis-supporting activity in serum from individual rats were not known, it was thought that, at least initially, in order to demonstrate meaningful differences between intact and thymectomized rat serum, serum pools would have to be used. Blood was obtained from eight intact male rats and six thymectomized male rats, see table 3.2. Each animal was bled by aortic puncture following ether anaesthesia, and, as far as possible, intact and thymectomized rats were bled alternately. The blood was kept at 4°C for eight hours before serum was obtained by centrifugation. The serum from each rat was placed in a sterile plastic bijou and stored at -20°C until used. The sera were used in three experiments: 3 (A), 3 (B) and 3 (C). On the day of each culture an intact and thymectomized rat serum pool was prepared by mixing equal volumes (500 μl. or 600 μl.) of serum from each sample. This meant that the serum used in experiments 3 (A), 3 (B) and 3 (C) had been thawed, at room temperature once, twice and three times respectively before use in that culture. The serum pools used in experiment 3 (C) were heat-inactivated.

Table 3.2 Details of the rats from which serum was obtained in order to prepare the intact and thymectomized rat serum pools and details of the storage of the serum before use in culture.

serum pool.	Rats.		Culture number:	3(A)	3(B)	3(C)
	number.	age when bled, days.	storage of serum before use in culture, days.			
Intact	4	131				
	4	105				
Tx	5	147		3	14	57
	1	106				

Experiment 3 (A) Comparison of the ability of pooled intact rat serum and pooled thymectomized rat serum to support PHA transformation in cultures of intact and thymectomized rat spleen cells.

Spleen cells were prepared from a 62-day-old intact rat and a thymectomized rat aged 46 days. Both rats had been exsanguinated prior to the removal of the spleen. The spleen cells were washed once by centrifugation, and were cultured in EBM containing 4 μl. PHA, erythrocytes washed six times by centrifugation and with 0 - 25% of serum from the intact or thymectomized rat pool. The results of this culture are shown in table 3.3 and figure 3.3.

When the amount of isotope incorporated by the intact and thymectomized rat spleen cells was adjusted for differences in cell number, then, for example, when no serum was added, a five-fold difference was found between the response of intact rat spleen cells and spleen cells from the thymectomized rat. The whole blood PHA responses of these rats were: intact rat 116, 017 cpm, thymectomized rat 58 cpm. The spleen cells from the thymectomized rat were therefore relatively more responsive to PHA than whole blood from the same rat.

Table 3.3 Isotope incorporation in cultures of intact rat spleen cells and thymectomized rat spleen cells stimulated by 4 μl. PHA in EBM supplemented with pooled intact or thymectomized rat serum.

Spleen cells. (number)	Serum %	Pooled rat serum.	
		Tx.	Intact.
		cpm mean ± SD.	
Intact rat spleen cells (0.8×10^6).	0	207,866 ± 10,964	207,866 ± 10,964
	0.2	220,194 ± 12,874	238,532 ± 14,179
	1	222,343 ± 25,107	232,285 ± 5,369
	5	144,167 ± 7,421	135,660 ± 9,350
	10	81,571 ± 8,093	69,887 ± 5,767
	25	34,655 ± 5,763	28,342 ± 6,724
Tx rat spleen cells (0.5×10^6).	0	24,721 ± 6,316	24,721 ± 6,316
	0.2	30,956 ± 3,865	42,279 ± 4,842
	1	19,785 ± 3,366	17,700 ± 2,484
	5	11,910 ± 2,104	13,578 ± 1,539
	10	11,070 ± 1,630	11,470 ± 909
	25	9,407 ± 1,502	10,134 ± 1,990

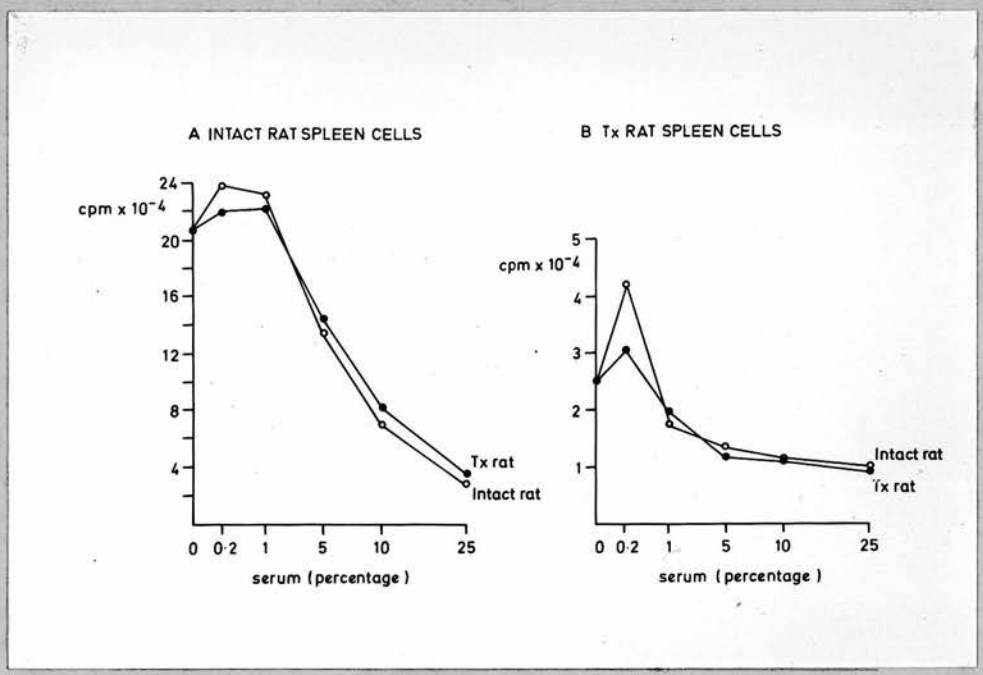


Figure 3.3 Comparison of the ability of pooled intact rat serum and pooled thymectomized rat serum to support transformation in response to 4 μl. PHA in cultures of (A) Intact rat spleen cells, (B) thymectomized rat spleen cells.

Experiment 3 (B) **Comparison of the ability of pooled intact rat serum and pooled thymectomized rat serum to support PHA transformation in cultures of washed intact rat blood cells and washed thymectomized rat blood cells.**

Blood was obtained by aortic puncture from a 77-day-old intact rat and a thymectomized rat aged 57 days. The blood cells were carefully washed in medium and saline by five centrifugations. The washed blood was cultured in EBM, 4 μl. PHA and pooled intact and thymectomized rat serum. The experimental design was identical to that used in the previous experiment (3A). The results are shown in table 3.4 and figure 3.4.

Table 3.4 Isotope incorporation in cultures of washed intact rat blood cells and washed thymectomized rat blood cells stimulated by 4μl. PHA in EBM supplemented with pooled intact or thymectomized rat serum.

Leukocytes (number).	Serum %	Pooled rat serum. Tx.	Pooled rat serum. Intact.
		cpm, mean ± SD.	
Washed intact rat blood (1.1×10^6)	0	162,981 ± 7,823	162,981 ± 7,823
	0.2	181,894 ± 7,136	183,701 ± 9,779
	1	191,032 ± 8,766	185,609 ± 1,945
	5	136,177 ± 7,315	135,626 ± 9,055
	10	86,984 ± 5,293	99,113 ± 9,991
	25	27,981 ± 5,392	29,270 ± 5,564
Washed Tx rat blood (0.3×10^6)	0	2,825 ± 738	2,825 ± 738
	0.2	3,241 ± 343	2,395 ± 460
	1	2,520 ± 294	2,129 ± 387
	5	2,083 ± 473	2,012 ± 1,648
	10	2,112 ± 187	1,711 ± 973
	25	2,691 ± 311	1,926 ± 382

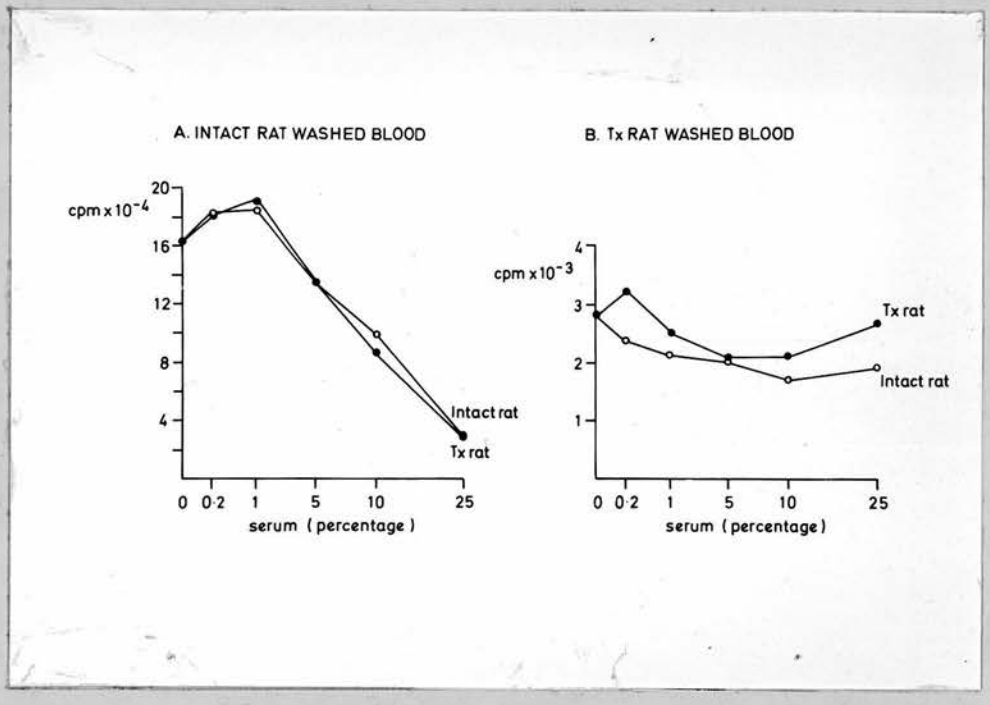


Figure 3.4 Comparison of the ability of pooled intact rat serum and pooled thymectomized rat serum to support transformation in response to 4 μl. PHA in cultures of (A) washed intact rat blood cells, (B) washed thymectomized rat blood cells.

Experiment 3 (C) Comparison of the ability of heat-inactivated pooled intact rat serum and pooled thymectomized rat serum to support PHA transformation in cultures of washed intact rat blood cells and washed thymectomized rat blood cells.

The experimental design was identical to that of the previous two cultures, except for the use of heat-inactivated serum and EMEM instead of EBM. The intact rat blood donor was 125 days old and the thymectomized rat blood donor was 99 days old. The results are presented in table 3.5 and figure 3.5.

The blood from the thymectomized rat, although not as reactive to PHA as the intact rat blood, was more responsive than was usual for a thymectomized rat. The histological check for a thymus remnant, however, was negative.

Table 3.5 Isotope incorporation in cultures of washed intact rat blood cells and washed thymectomized rat blood cells stimulated by 4 μl. PHA in EMEM supplemented with heat-inactivated pooled intact or thymectomized rat serum.

Leukocytes (number).	Serum %	Heat-inactivated pooled rat serum	
		Tx	Intact
		cpm, mean ± SD.	
Washed intact rat blood (0.97×10^6)	0	163,169 ± 7,512	163,169 ± 7,512
	0.2	189,567 ± 5,992	188,078 ± 19,766
	1	207,490 ± 5,688	200,024 ± 4,644
	5	184,782 ± 12,332	170,148 ± 5,451
	10	140,554 ± 16,771	136,785 ± 4,370
	25	54,177 ± 1,821	43,620 ± 10,722
Washed Tx rat blood (0.76×10^6)	0	54,686 ± 1,732	54,686 ± 1,732
	0.2	84,422 ± 11,032	84,153 ± 1,191
	1	77,165 ± 4,378	98,989 ± 20,012
	5	66,148 ± 4,464	60,251 ± 19,300
	10	33,345 ± 1,814	29,481 ± 1,337
	25	8,226 ± 2,977	4,319 ± 441

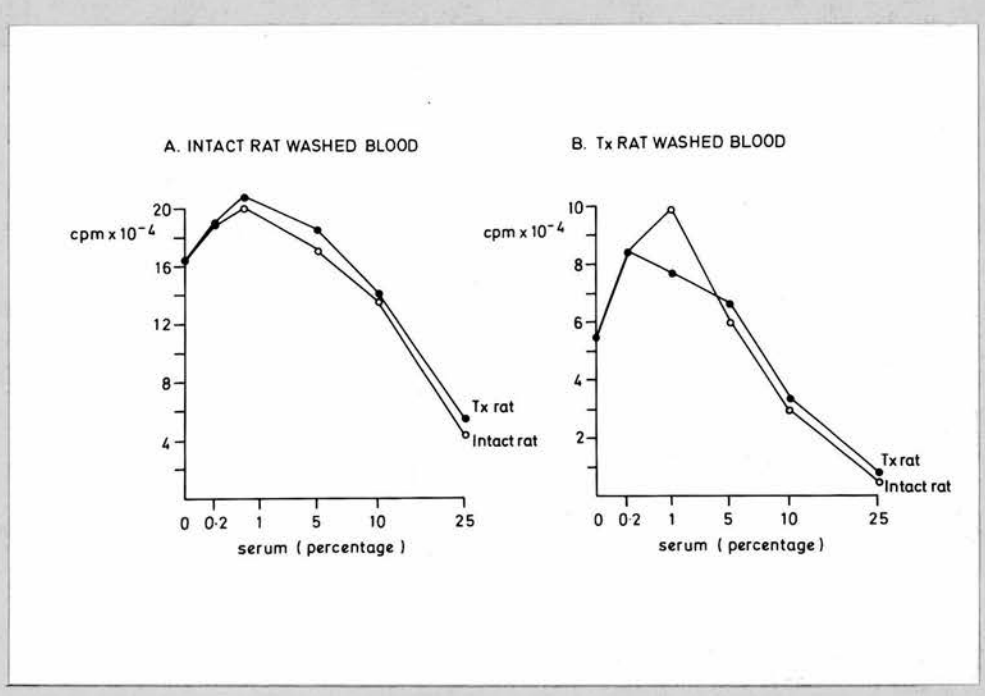


Figure 3.5 Comparison of the ability of heat-inactivated, pooled intact rat serum and heat-inactivated, pooled thymectomized rat serum to support transformation in response to 4 μl. PHA in cultures of (A) washed intact rat blood cells, (B) washed thymectomized rat blood cells.

Experiment (4) <u>The effect of fresh, intact and thymectomized rat plasma on tritiated-thymidine incorporation in PHA-stimulated and unstimulated thymectomized rat spleen cell cultures</u>.

In all previous experiments described in this chapter the serum had been stored at -20°C for 3 - 54 days and had been thawed at least once before being used. As it was possible that the hypothetical activities in the serum that were under investigation might be unstable when stored frozen and subsequently thawed, it was decided that fresh rat plasma should be studied.

An intact male rat and a thymectomized male rat of the same age (158 days) were bled from the aorta within five minutes of each other. Approximately the same quantity of blood, from each rat, was put into heparin tubes and the tubes were placed in a refrigerator. Within 50 minutes of the blood having been taken, plasma was obtained by centrifugation and was kept in the refrigerator until used. The plasma was added to the cells within one and a half hours and the culture vials placed into the incubator two hours after the rats had been bled. The plasma donors had been bled, 15 and 5 weeks previously and their whole blood PHA responses on these occasions were found to be typical of intact: 85,957 and 86,869 cpm., and thymectomized: 18,271 and 3,871 cpm. rats.

The spleen cells were prepared from the same thymectomized rat that donated the plasma. 18.3×10^6 spleen cells were placed into plastic universal containers with 6.6 ml. EMEM supplemented with either 0%, 1%, 5% or 20% fresh intact or thymectomized rat plasma. The universal containers were placed, loosely capped, at an angle of about 30° to the horizontal, for 17 hours in the plastic box, at 37°C, in an

atmosphere of 5% CO_2 in air. After 17 hours, the universal containers (UC's) were immediately put on ice and, in turn, each UC was centrifuged twice at 2,000 rpm for 10 minutes. For half of the UC's - those designated "continuous culture", no medium was removed. The remaining UC's, designated "preculture to fresh", had 6 ml. of medium removed after each centrifugation and 6 ml. of fresh EMEM (containing no plasma) added back. The cells were then resuspended by gentle agitation and 1 ml. aliquots made into vials containing either 4 µl. PHA in 100 µl. saline or 100 µl saline. 100 µl. of a thoroughly washed erythrocyte preparation was then added. The remainder of the culture followed the standard procedure. The culture was terminated 72 hours after the addition of PHA and 20 hours after the addition of the isotope.

The results are shown in tables 3.6 and 3.7. A comparison between PHA-stimulated DNA synthesis in "continuous culture" and "preculture" is shown in figure 3.6. DNA synthesis, in the absence of PHA, is compared in the continuous presence of plasma to that where plasma was removed after 17 hours, in figure 3.7.

The high isotope incorporation by the thymectomized rat spleen cells is accounted for by the greater number of cells per culture than in other experiments in this chapter.

Table 3.6 Response of 2.8×10^6 spleen cells from a thymectomized rat to 4 µl. PHA after a 17 hour culture in EMEM containing various concentrations of fresh plasma from an intact or thymectomized rat followed by culture in fresh EMEM without plasma (preculture to fresh) or in unchanged medium (continuous culture).

Type of culture.	Plasma concentration %	Fresh rat plasma.	
		Tx	Intact
		cpm, mean ± SD.	
"Preculture to fresh"	0	121,851 ± 14,890	121,851 ± 14,890
	1	144,090 ± 5,143	156,431 ± 7,017
	5	159,904 ± 12,725	159,348 ± 3,772
	20	102,934 ± 7,387	146,391 ± 5,912
"Continuous"	0	173,990 ± 27,697	173,990 ± 27,697
	1	176,696 ± 6,841	192,026 ± 2,967
	5	97,012 ± 1,444	137,485 ± 6,375
	20	34,511 ± 6,690	44,989 ± 1,184

Table 3.7 Isotope incorporation in cultures of 2.8×10^6 spleen cells from a thymectomized rat in the absence of PHA after a 17 hour culture in EMEM containing various concentrations of fresh plasma from an intact or thymectomized rat followed by culture in fresh EMEM without plasma (preculture to fresh) or in unchanged medium (continuous culture).

Type of culture.	Plasma concentration %	Fresh rat plasma.	
		Tx	Intact
		cpm, mean ± SD.	
"preculture to fresh".	0	206 ± 14	206 ± 14
	1	393 ± 59	416 ± 67
	5	309 ± 43	555 ± 21
	20	286 ± 37	513 ± 81
"continuous".	0	143 ± 12	143 ± 12
	1	350 ± 40	426 ± 63
	5	354 ± 14	833 ± 32
	20	125 ± 17	586 ± 290

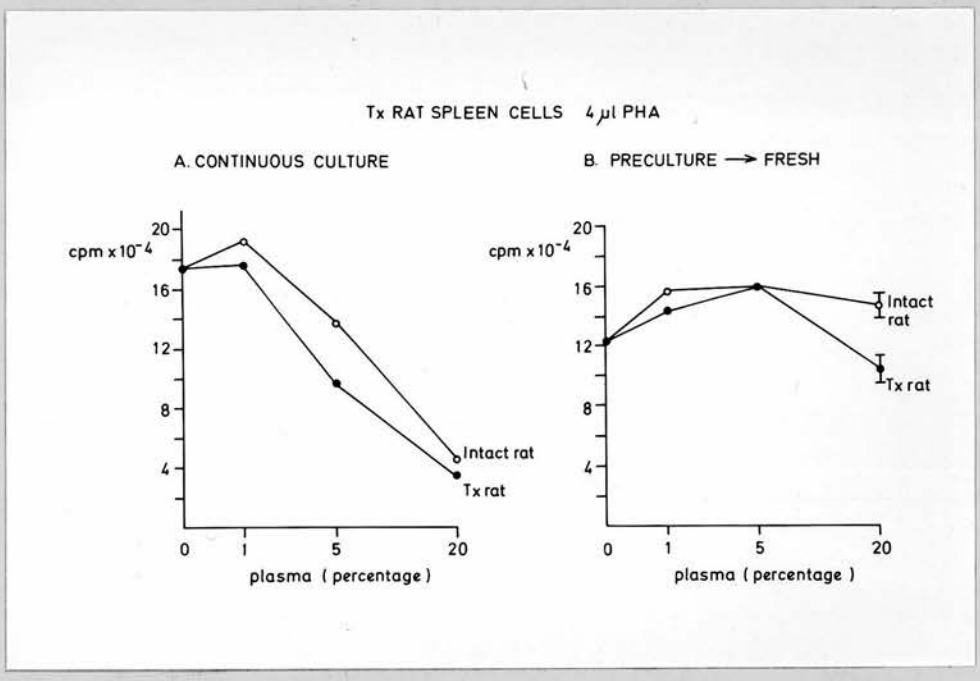


Figure 3.6 Comparison of the ability of fresh intact rat plasma and fresh thymectomized rat plasma to effect tritiated thymidine uptake in cultures of 2.8×10^6 thymectomized rat spleen cells. 4 µl. PHA was added to all culture vials after 17 hours. The cells were either (A) cultured in the continuous presence of plasma (continuous culture) or, (B) resuspended in medium without plasma, after a 17 hour preculture (preculture to fresh). Vertical lines represent plus and minus one standard deviation from the mean of triplicate cultures.

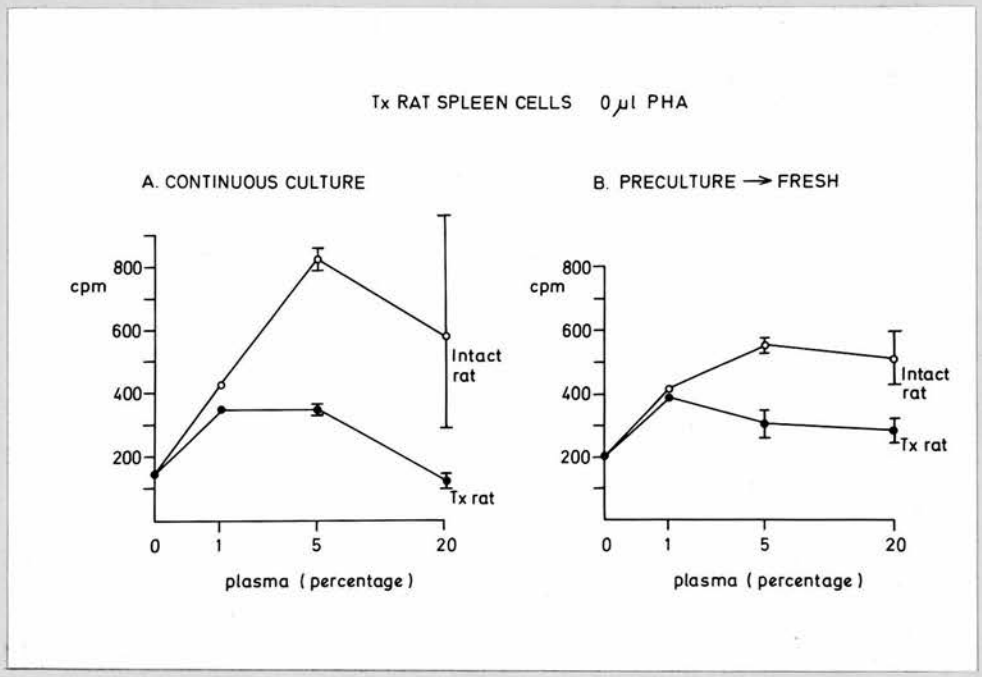


Figure 3.7 Comparison of the ability of fresh intact rat plasma and fresh thymectomized rat plasma to effect tritiated thymidine uptake in cultures of 2.8×10^6 thymectomized rat spleen cells in the absence of PHA. The cells were either (A) cultured in the continuous presence of plasma (continuous culture) or, (B) resuspended in medium without plasma after a 17 hour preculture (preculture to fresh). Vertical lines represent plus and minus one standard deviation from the mean of triplicate cultures.

CONCLUSIONS AND DISCUSSION.

Tritiated-thymidine incorporation was found to decrease, in cultures of lymphocytes stimulated by PHA, as the concentration of serum or plasma in the medium increased. Heat-inactivation was thought to be responsible for reducing the depressive activity of rabbit serum but was found to have little effect on pooled rat serum. No difference was found between the ability of intact and thymectomized rat serum, whether heat-inactivated or not inactivated, to depress isotope incorporation in PHA-stimulated spleen and washed blood cultures. It is therefore unlikely that the origin of the serum immuno-suppressive factor is the thymus or T cells. The experiments described in this chapter do not, however, represent an exhaustive investigation. For example, only one PHA concentration was used, that of 4 μl. per culture, a previously determined optimum for intact rat whole blood cultures. It is possible that other PHA concentrations and other variations in the culture method might have led to the obtainment of positive results.

The addition of serum or plasma to the medium was not found to be necessary in order to obtain a successful response to PHA. This result is not in agreement with that reported by Pauly and co-workers who found very little reactivity to PHA in thoroughly washed human blood cultures (Pauly, J.L. et al 1973). Unpresented results from three washed human blood cultures showed a response, in the absence of serum, of between 10 and 15% of the response in the presence of 10% serum. It is not clear why rat blood cultures were more successful in the absence of serum than cultures of human blood.

An unexpected finding was the very large variation in the ability of samples of serum from different rats to support PHA-induced lymphocyte transformation. The degree of variation was unexpected

because the rats were age-matched as well as genetically identical. The variation might have been the result of biological and technical variations in the culture and in the assessment of the response to PHA. Also, differences in the manipulation of the sera could have contributed, although each blood sample was treated as nearly identically as possible, to each other. All the donor rats appeared to be in good health, however, unnoticed illness, natural physiological variation and stress also undoubtedly contributed to the variation in DNA synthesis supporting activity. A similar result was found by Mangi and co-workers. They reported a 10-fold variation in the response of normal human lymphocytes to PHA when the cells were cultured in medium containing 15% plasma from 26 samples, which had been stored for 2 - 3 months at -70°C (Mangi, R.J. et al 1974). Mangi obtained similar results with fresh serum. Also human peripheral blood lymphocytes have been shown to respond variably to PHA in the presence of serum taken at different times from the same donor (McIntyre, O.R. and Cole, A.F. 1969).

The experiment comparing intact and thymectomized rat plasma showed suggestive differences at one or two plasma concentrations in the presence and absence of PHA. As this was an experiment using plasma from individual donors and was not repeated positive results cannot be reported for differences between the plasma of intact and thymectomized rats. The preculture experiment showed that fresh plasma was not toxic, as cells, once washed free of most of the varying quantities of plasma, responded to PHA to more or less the same extent. In cultures without PHA, incorporation of tritiated thymidine was always greater in medium containing plasma from the intact rat. This latter finding, although worthy of further investigation, was not followed up.

With regard to the possible serum factor increasing the response of lymphoid cells to PHA, no convincing difference was found between intact and thymectomized rat serum or plasma. There are two explanations to account for this, either such factors do not exist or the test system was inadequate to demonstrate these activities. The experiment in which the lymphocytes were precultured with plasma for 17 hours was thought to be the one most likely to reveal such a factor, as the cells would have had a chance to undergo modification before PHA was added. In this experiment it was found that at one or two plasma concentrations uptake of isotope was greater in cultures containing intact rat plasma than in cultures containing plasma from the thymectomized rat. As this experiment was not repeated, the demonstration of a factor in the plasma of intact rats increasing the isotope uptake in lymphoid cells was not conclusive.

CHAPTER FOUR

IN VIVO STUDIES.

INTRODUCTION

Results from preliminary studies suggesting the thymic dependence of the whole blood lymphocyte response to PHA have been presented in chapter two.

In this chapter further evidence that the response to PHA is a function of T cells is presented and the suitability of whole blood PHA transformation as an assay to measure immune restoration is assessed. This was done by measuring the restoration of the response to PHA in thymectomized rats following the grafting of syngeneic thymuses. The mechanism of the restoration of immunocompetence by thymus transplantation has been discussed fully in chapter one, where both the cellular and humoral contributions to restoration have been considered.

Thymus grafting has been shown by previous workers to restore, either partially or completely, the response of thymus-deprived animals to PHA (Dukor, P. and Dietrich, F.M. 1967) (Davies, A.J.S. et al 1968) (Stutman, O. 1970) (Dabrowski, M. et al 1970) (Doenhoff, M.J. et al 1970) (Takiguchi, T. et al 1971 a), and to increase to normal levels the response to PHA in patients with DiGeorge's syndrome (August, C.S. et al 1968) (Cleveland, W.W. et al 1968) (Kazimiera, J. et al 1973).

A whole blood PHA test has several advantages over the various techniques for lymphoid cell culture. Subject to surviving cardiac puncture, the animal can be experimentally manipulated and at a later stage another whole blood PHA culture performed. Serial assessments of immunocompetence can therefore be performed on the same animal and more direct comparisons made. As not more than one ml. of blood need be taken from each rat, for each assay, the physiological consequences of such blood losses are slight. Also, because fewer procedures need to be carried out on the whole blood sample more experimental variables can be studied in the same experiment.

EXPERIMENTAL DESIGN

A diagrammatic representation of the design of the two experiments is shown in figure 4.1.

Reconstitution Experiment 1 sought to confirm that neonatal thymectomy led to a persistant lymphopenia and a markedly depressed whole blood response to PHA. On the day following the first whole blood culture, half of the surviving thymectomized rats were given subcutaneous thymus grafts and the other half remained untreated. Further PHA tests were done on all the rats, after four and eleven weeks, in order to investigate the time scale and the magnitude of any restoration of the response to PHA brought about by thymus transplantation.

Reconstitution Experiment II sought to investigate a possible humoral action of the thymus by studying the effect of injecting thymectomized rats with cell-free medium obtained from thymus organ cultures. Blood lymphocyte counts and the response to PHA in the organ medium treated rats were compared to those of untreated thymectomized rats, thymectomized rats bearing thymus grafts and untreated intact rats.

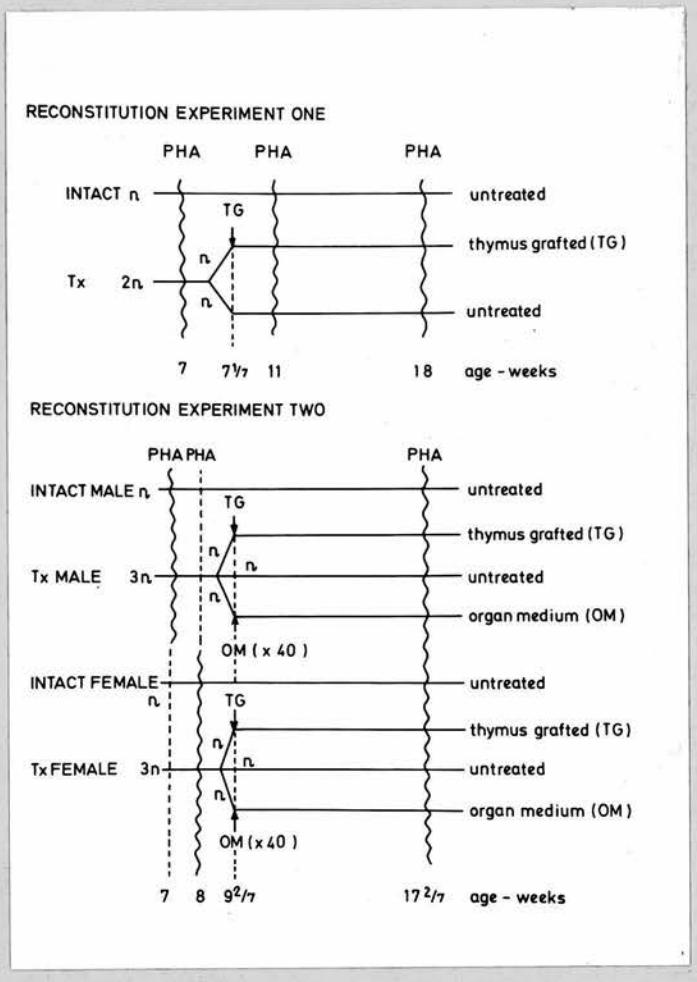


Figure 4.1 **The design of Reconstitution Experiments 1 and II.**

RECONSTITUTION EXPERIMENT ONE

A breeding programme was arranged to produce sufficient rats, born within 48 hours of one another, such that at seven weeks of age a group of 24 neonatally thymectomized rats and one of 12 sham-thymectomized rats was available. These groups consisted of 6 intact male, 6 intact female, 13 thymectomized male and 11 thymectomized female rats.

When the rats were seven weeks old the first whole blood PHA culture was performed. Each rat was anaesthetized and bled by cardiac puncture in an order such that every third rat bled was an intact rat. The precordial area of each rat was shaved and cleaned with 70% alcohol. Approximately 1 ml. of blood was taken from each rat using a heparinised 2 ml. syringe and a 21 gauge microlance. The blood was thoroughly mixed whilst in the syringe and carefully transferred to a numbered sterile plastic vial. After having been bled the rats were returned to different cages so as not to come into contact with those rats which had not yet been bled. Standard whole blood cultures were performed using EBM and 4 μl. PHA. Assessment of the response to PHA was made using tritiated thymidine of specific activity, 23 Ci/m.mol. for the first two cultures and tritiated thymidine of specific activity 27 Ci/m.mol. for the third culture. Total WBC counts and blood smears were made on all blood samples, as soon as possible, between one and ten hours after initiation.of the culture.

On the day following the first bleeding and therefore before the result of the first culture was known, the 18 surviving thymectomized rats were divided into two groups of 9 rats. The rats were allocated to each group by random selection with the provision that the sex ratio was kept as constant as possible. One group was then thymus grafted. Thymuses from two ADRI donor rats, aged 6 - 18 days, were cut

into pieces of about 1 - 2 mm^3 and placed into a subcutaneous pocket prepared on the back of the recipient rat. The thymus donors were sexed before sacrifice and in eight out of the nine grafted rats, the sex of the thymus donor matched that of the recipient.

Four weeks and eleven weeks after the first whole blood culture, the surviving rats from the three experimental groups: (1) sham-thymectomized, (2) thymectomized, untreated, (3) thymectomized, thymus grafted; were bled, whole blood PHA cultures performed and total and differential white cell counts made.

Results.

The survival rate in the three sessions of cardiac puncture was 72%, 85% and 77% respectively. The rats that died, died shortly after bleeding, and those surviving, recovered within a few hours from any visible ill effects of the experience. It was found that, in some of the rats in which difficulty was experienced in obtaining blood, an unexpectedly low total white cell count and PHA response occurred. Results from the few such samples were discarded.

At the end of the experiment, or whenever a thymectomized rat died, an autopsy was performed to check for the possible presence of a thymic remnant and a thymus graft, when relevant. A thymus graft was recovered from only two of the nine thymus-grafted rats, four and fifteen weeks respectively after grafting. A photomicrograph of the thymus graft recovered after 15 weeks is shown in figure 4.2.

It is well documented that neonatal thymectomy in rodents leads to a persistent lymphopenia. Results from this study confirm that, in the rat, neonatal thymectomy is associated with a significant reduction in the total white blood cell count which is caused by a reduction in the

number of circulating lymphocytes. The blood picture of the group of untreated intact and thymectomized rats at seven weeks of age is shown in table 4.1. Results similar to these were found when the rats were bled at eleven and eighteen weeks. Concomitant with the decreased blood lymphocyte count in the group of thymectomized rats was a large reduction in whole blood response to PHA. A comparison of blood lymphocyte counts and whole blood PHA responses during the three cultures is shown in figures 4.3 and 4.4. The PHA response of the untreated experimental rats at seven weeks of age is shown in figure 4.5. In order to decide whether differences between the whole blood PHA response of intact and thymectomized rats were due simply to differences in total lymphocyte number, the isotope uptake for each blood sample was arithmetically adjusted to take account of the number of lymphocytes per sample. A comparison of the isotope incorporation per 10^6 lymphocytes of intact and thymectomized rat blood samples is shown in figure 4.6. These results indicate that qualitative as well as quantitative differences exist between lymphocytes from thymectomized and intact rats with respect to their ability to respond to PHA.

In order to see what relationship existed between the number of lymphocytes per culture and the response to PHA, plots of total lymphocyte number against isotope incorporation were constructed. These plots are reproduced in figure 4.7. Especially from the results obtained from the second and third cultures, a linear relationship appeared to exist between the number of lymphocytes per culture and isotope incorporation. Lines of best fit were constructed from linear regression analyses and extrapolated to cut the lymphocyte axis at the point where no isotope uptake would occur. The results obtained from such analysis is shown in table 4.2. There are several reasons why a linear relationship between lymphocyte number and isotope incorporation might break down.

A major assumption is that the number of cells at the onset of culture is proportional to the number of cells surviving and incorporating isotope in response to PHA. At cell densities above a certain point the tritiated thymidine might not be present in saturating concentrations and thus a non-linear uptake of isotope would result. These and other related points have been discussed in chapter two. A linear relationship would demand that all the rats had very similar unresponsive cell populations. In fact, the thymectomized rats might have increased numbers of B cells due to infections or to partly compensate for a deficiency of T cells. However, assuming a linear relationship, the value where the constructed line (figure 4.7) cut the lymphocyte axis was taken to be the number of unresponsive lymphocytes per culture. Table 4.2 contains a compilation of the values for the unresponsive cell population calculated from the results of the three whole blood cultures. This cell population for reasons discussed in chapter two, was thought to be the B cell population. Over the three whole blood cultures, the B cell population and the percentage of B cells in the blood of the intact rats was very similar.

A major difficulty in assessing the degree of restoration of the response to PHA was the "natural" variation in PHA response, illustrated by results from the untreated rats, figure 4.4. At no time during the experiment did any "spontaneous" restoration of the PHA response of an untreated thymectomized rat occur. Six out of nine thymus-grafted rats showed increased PHA responses four weeks after grafting. Eleven weeks after grafting, two of the three rats that showed no restoration at four weeks showed PHA responses in the range of those found for the intact rats, whilst three out of the six rats that showed increased responses at four weeks showed either a stabilized PHA response or a decreased response from that found four weeks after grafting. In summary, eight out of the

nine thymus-grafted rats showed an increased PHA response of an order of magnitude not seen in untreated thymectomized rats. At four weeks after grafting, one rat, and eleven weeks after grafting, three rats, had PHA responses in the range of those of the intact group.

Comparison of lymphocyte counts for the three groups over the three cultures, figure 4.3, showed a similar pattern to that seen with the PHA responses. One or two rats had restored lymphocyte counts four weeks after grafting and four rats had restored counts eleven weeks after grafting. When the lymphocyte number and PHA response of individual thymus-grafted rats was compared, a general finding was that those rats which had increased numbers of lymphocytes were the rats that showed a greater restoration of PHA response. This association was not always found, however, perhaps because of some inaccuracy in blood lymphocyte counts.

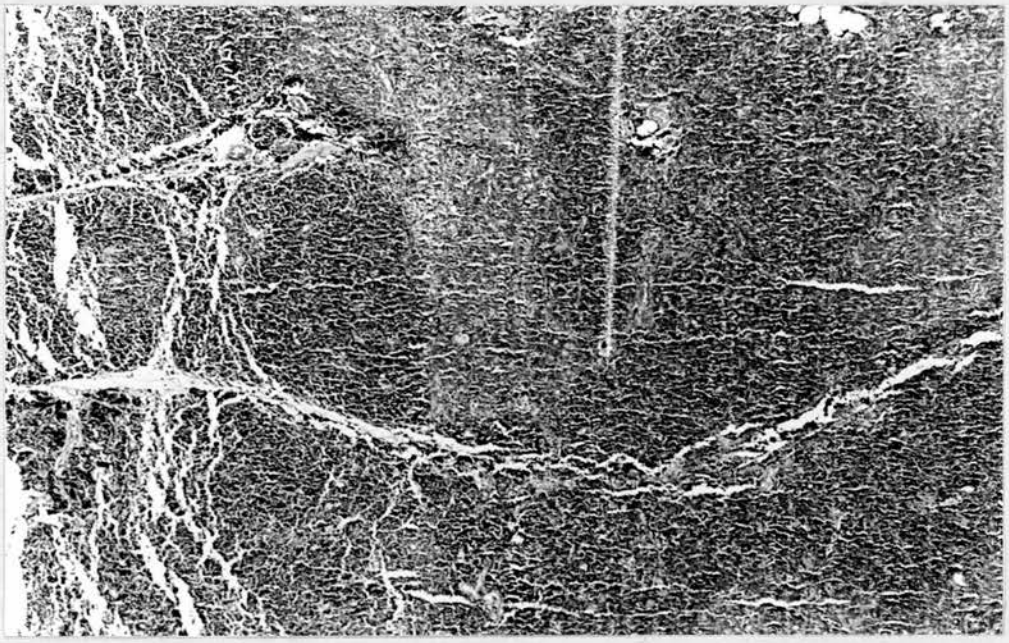


Figure 4.2 Thymus graft placed subcutaneously and removed after 15 weeks (x 50).

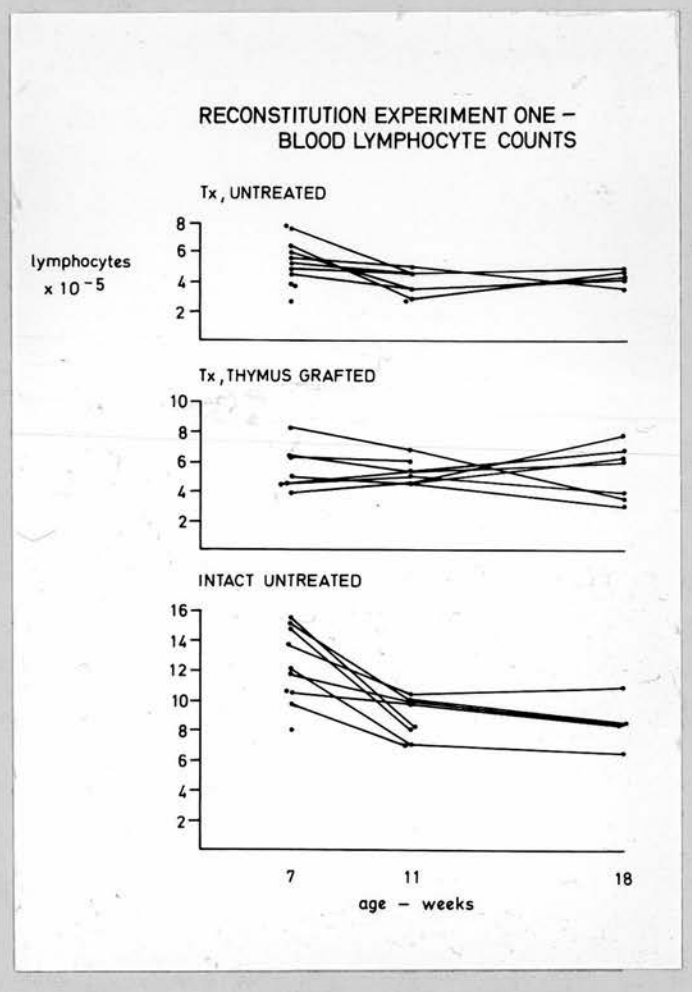


Figure 4.3 Blood lymphocyte counts of individual Tx, untreated; Tx, thymus grafted and untreated intact rats at 7, 11 and 18 weeks, Reconstitution Experiment 1.

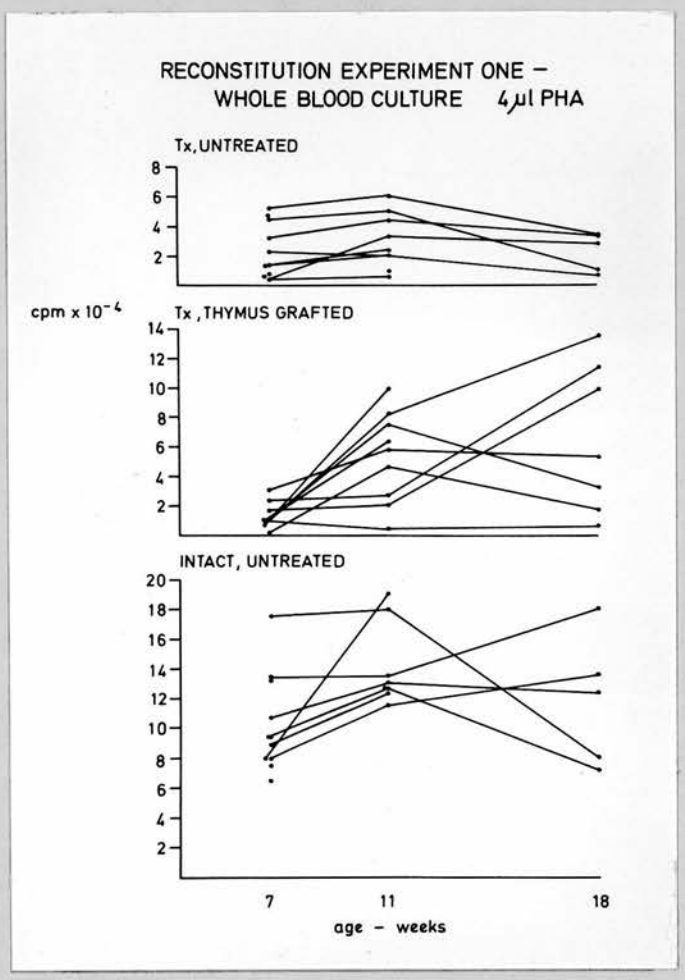


Figure 4.4 Whole blood PHA response of individual Tx, untreated; Tx, thymus-grafted and untreated intact rats at 7, 11 and 18 weeks, Reconstitution Experiment 1.

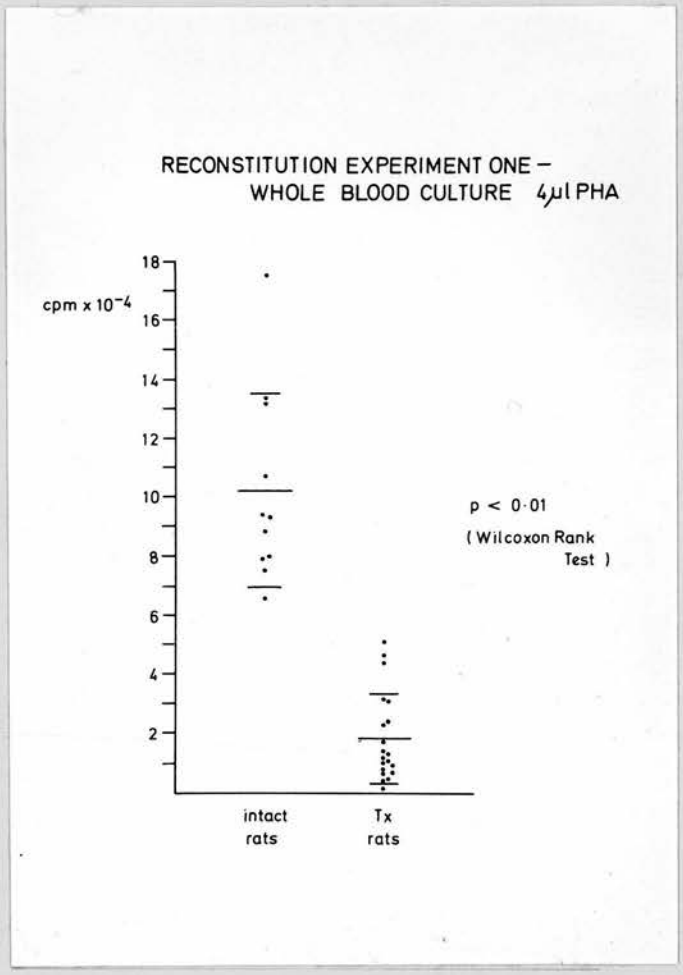


Figure 4.5 Whole blood PHA response of untreated intact and thymectomized rats at 7 weeks of age, Reconstitution Experiment 1. Horizontal lines represent mean and standard deviations.

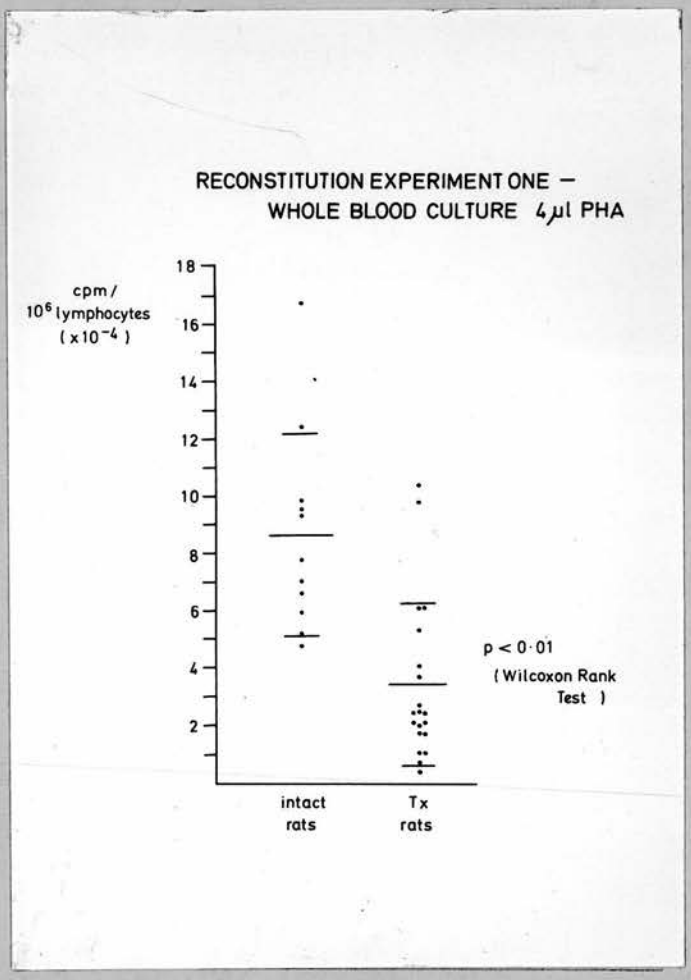


Figure 4.6 Whole blood PHA response of untreated intact and thymectomized rats at 7 weeks of age expressed as cpm per 10^6 lymphocytes, Reconstitution Experiment 1. Horizontal lines represent mean and standard deviations.

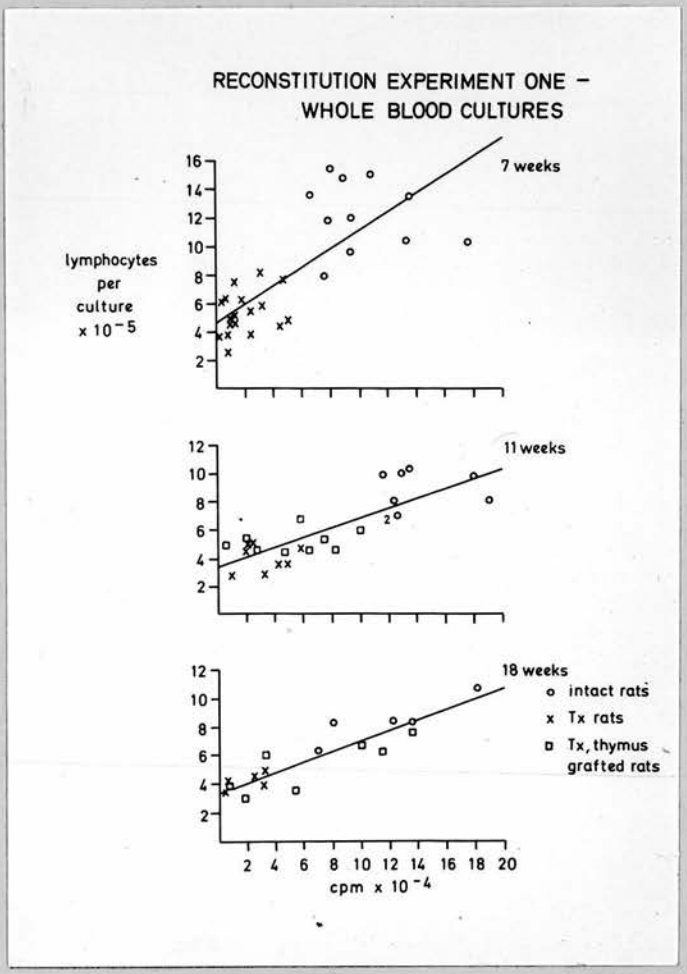


Figure 4.7 Plots of lymphocyte number and isotope incorporation in PHA stimulated whole blood samples from intact, thymectomized and thymectomized-thymus grafted rats at 7, 11 and 18 weeks, Reconstitution Experiment 1.

Table 4.1 Blood picture of group of intact and thymectomized rats at seven weeks of age, Reconstitution Experiment 1.

Rats (number)	Mean ±S.D.		%	Mean ±S.D.	%	Mean ±S.D.	%
	Total WBC /mm^3.	Total Lym. /mm^3.	Lym.	Total Poly. /mm^3.	Poly.	Total Mono. /mm^3.	Mono.
Intact (12)	13,273 ±2,638	12,341 ± 2,426	93.0	798 ± 368	6.0	134 ± 91	1.0
Tx. (20)	6,468 ± 1,711	5,298 ± 1,466	81.9	1,066 ± 533	16.5	103 ± 47	1.6
Wilcoxon rank test	$p < 0.01$	$p < 0.01$	-	*ND	-	ND	-

* ND - not done.

Table 4.2 Results obtained from linear regression analysis of the data in figure 4.7, in which the lymphocyte number and PHA response of individual blood samples over three cultures are compared.

Age of rats at culture (weeks).	Mean lymphocyte count of intact rats. per 0.1 ml. ($\times 10^{-2}$)	Mean number of unresponsive lymphocytes per culture. ($\times 10^{-2}$)	Mean percentage unresponsive cells in blood of intact rats.	Gradient ($\times 10^{-1}$)	Coefficient of linear regression (r)
7	12,341	4,694	38.0	0.6446	0.7741
11	8,768	3,368	38.4	0.3454	0.8123
18	8,433	3,372	40.0	0.3677	0.9001

RECONSTITUTION EXPERIMENT TWO

The design of this experiment, shown in figure 4.1, was similar to that of Reconstitution Experiment 1. An addition group was included, thymectomized rats treated with injections of medium obtained from thymus organ cultures. In order to accommodate this extra group and still keep the numbers experimentally managable, the male and female rats were bled, initially, on successive weeks. Despite a much higher survival rate, an overall 90% surviving cardiac puncture, the PHA response of all rats, both male and female, was compared in the third and final culture.

At seven weeks of age the male rats (20 thymectomized, 9 intact) were bled and the first PHA culture performed. At eight weeks the female rats (18 thymectomized, 8 intact) were similarly bled. The method of bleeding and setting up the culture was the same as that described in Reconstitution Experiment 1. Assessment of the response to PHA, in Reconstitution Experiment II was done using tritiated thymidine of specific activity 5 Ci/m.mol.

Nine days after the female rats had been bled, when the result of the initial PHA cultures were known, the 34 surviving thymectomized and 13 intact rats were placed into four experimental groups as follows: (1) intact rats; (2) thymectomized, untreated rats; (3) thymectomized thymus-grafted rats; (4) thymectomized rats injected with tissue culture medium from thymus organ cultures.

The donors of the thymuses used for grafting were obtained from three litters of ADRI rats aged 4, 13 and 14 days respectively. As far as possible the sex of the thymus donor was matched with the sex of the recipient, except in the case of the four day old rats which were not sexed. The four day old rats were killed by decapitation and the older

rats by ether narcosis. The thymuses were placed in cold saline and cut into pieces about 1 - 2 mm^3. These pieces were then placed under the capsule and in the tissue surrounding the left kidney, using the method described in chapter two.

Thymus organ culture was carried out by techniques that are fully described in chapter six. In brief, six pieces of thymus, sized about 1 mm^3, obtained from ADRI male rats aged between 54 - 68 days were supported on a stainless steel grid and cultured in medium 199 supplemented with bicarbonate, glutamine, antibiotics, 5 mM HEPES and 5% fresh male ADRI rat serum. The petri dishes in which the cultures were performed contained about 6 ml. medium. After culture for three days at 37°C in a humidified atmosphere of 5% CO_2 in air the medium was harvested and replaced by fresh medium. After a further three days, the medium was again harvested and the culture terminated. The harvested medium was pooled, centrifuged for 10 minutes at 2,000 rpm at room temperature and the supernatant stored at 4°C until used. During an eight week period, each rat in the organ medium treated group was injected 40 times with thymus organ cultured medium, and nine organ cultures (over 200 dishes) were used to prepare the approximately two litres of medium needed for injection. Due to the almost continuous preparation of thymus organ culture medium, the medium was usually injected within three days after it had been harvested. The schedule of injections began on the same day that the group of thymectomized rats was thymus grafted. During eight weeks, six male and six female rats were injected subcutaneously 40 times. Each male rat received 5 ml. in each injection and the females received a group weight-corrected volume ranging from 3.2 ml. at the start to 2.8 ml. at the end of the treatment. The medium was usually injected into two separate sites on the ventral surface of the male rats and into one site for the female rats.

Eight weeks after the thymus grafting and the beginning of the schedule of injections, PHA whole blood cultures were done on the surviving rats from all four groups.

Results.

Within six weeks of the final PHA culture all the experimental rats were killed and autopsied. No thymic remnants were found in any of the thymectomized animals. In five out of the ten thymus-grafted rats, thymus grafts were recovered and confirmed by histology. An example of a thymus graft removed 12 weeks after transplantation is shown in figure 4.8.

The mean weights of the rats of the four experimental groups is shown in figure 4.9. It can be seen that neither thymectomy nor any subsequent manipulation had any marked effect upon the weight gains of the rats.

Results from Reconstitution Experiment II confirmed all the findings of Reconstitution Experiment 1. For example, the persistant lymphopenia in the group of untreated thymectomized rats and the partial restoration of the blood lymphocyte count in thymus grafted rats is shown in figure 4.10. The depressed whole blood PHA response in thymectomized rats and the partial restoration of response in thymus-grafted rats is shown in figure 4.11.

The rats belonging to the thymectomized, organ medium treated group were, with regard to blood lymphocyte counts and whole blood PHA response, no different to those rats in the untreated thymectomized group. This is clearly shown in figures 4.10 and 4.11. Injection of thymus organ medium had no discernable effect on the blood picture of the thymectomized rats. This is shown in table 4.3 where the blood picture of the rats

from all four groups at the time of the final culture is compared.

One of the thymectomized female rats was bled, by mistake, along with the male rats. When this error was realised the rat was put into the group of organ medium treated female rats. After two weeks of organ medium injections this female rat gave birth to a litter of six, which was removed and killed shortly after it was discovered. This animal was nevertheless, given the remaining injections along with the other females. No difference in the whole blood PHA response or lymphocyte count was found between this rat and the other thymectomized rats. This finding was of interest in view of the reported immunological recovery in thymectomized rodents following pregnancy (see chapter one). A "double dose" of possible thymus replacement factors had thus failed to bring about any restoration.

Plots of lymphocyte number against isotope incorporation for the three cultures are shown in figure 4.12. These plots were analysed in the same manner as those from Reconstitution Experiment 1, and the results obtained are shown in table 4.4. The values for the calculated number of unresponsive cells per culture and the percentage of unresponsive cells in the blood of the intact rats were again very similar and also similar to the values found in Reconstitution Experiment 1.

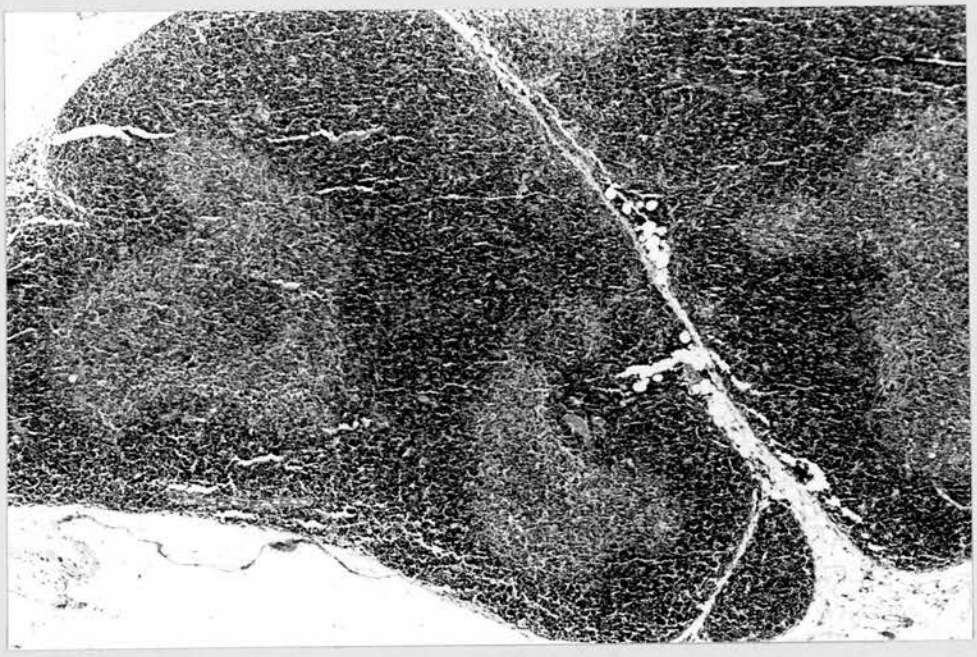


Figure 4.8 Thymus graft placed beneath the kidney capsule and removed after 12 weeks (x 50).

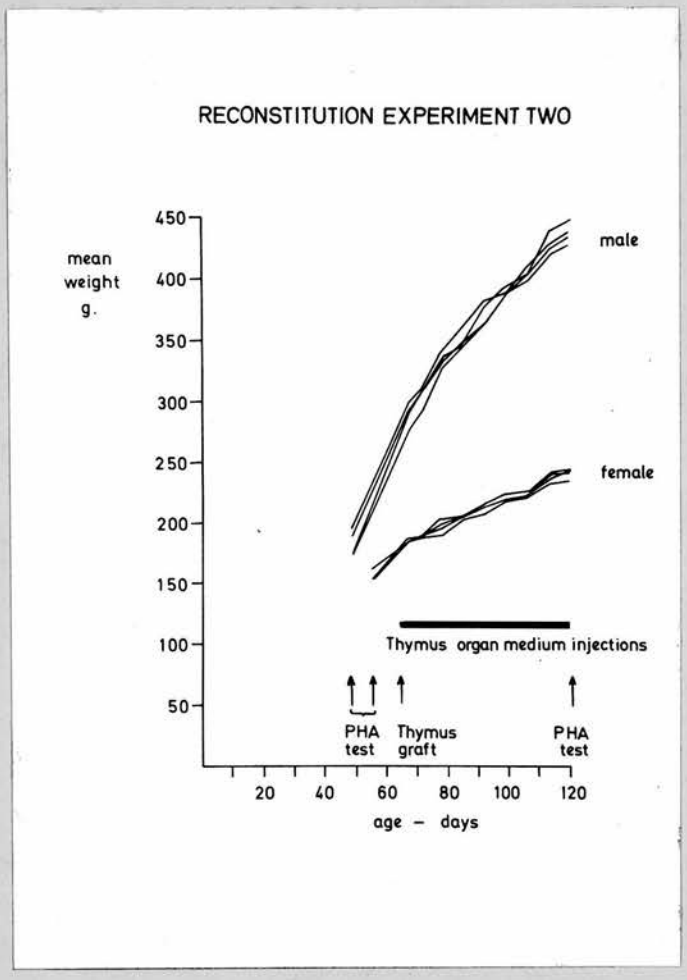


Figure 4.9 **Mean weight gains of the rats from the four experimental groups: intact untreated, thymectomized untreated, thymectomized thymus grafted and thymectomized organ medium treated. Reconstitution Experiment II.**

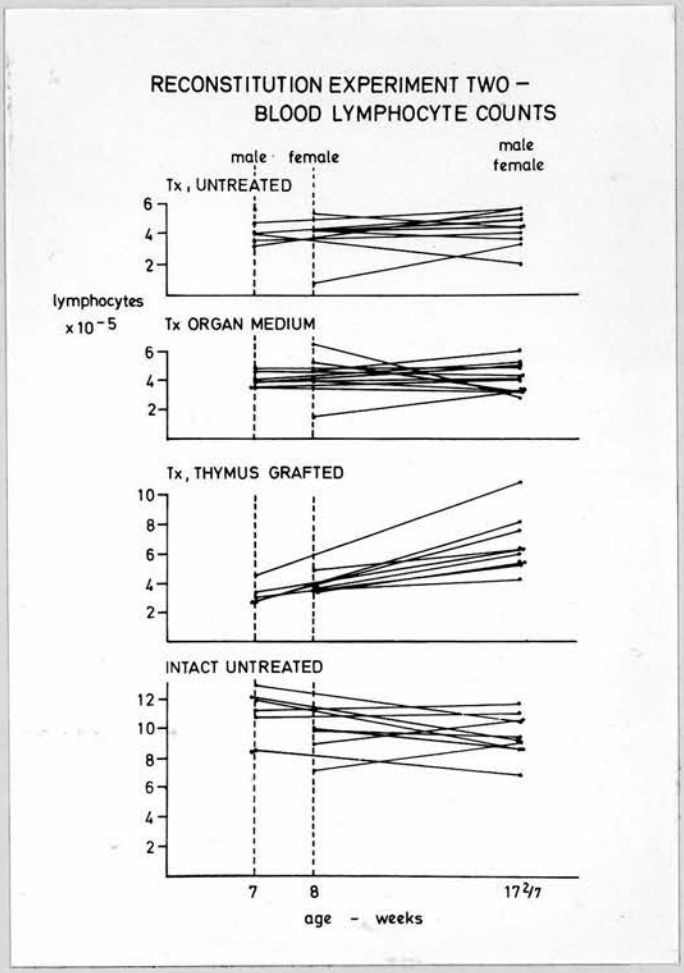


Figure 4.10 Blood lymphocyte counts of individual Tx, untreated; Tx, organ medium treated; Tx, thymus grafted and untreated intact rats at 7, 8 and 17 $^{2}/7$ weeks, Reconstitution Experiment II.

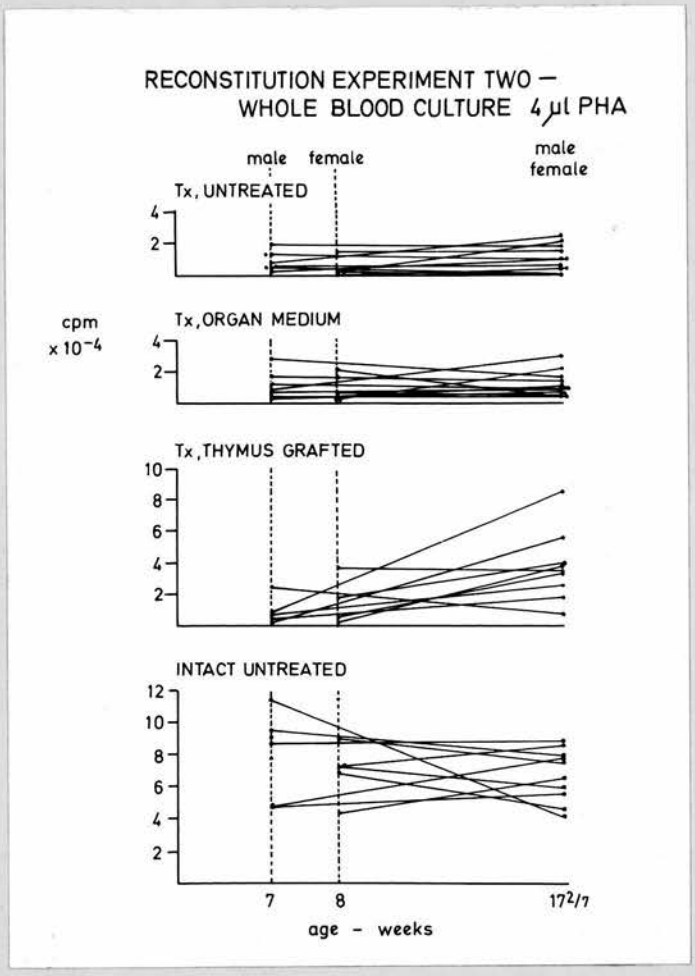


Figure 4.11 Whole blood PHA response of individual Tx, untreated; Tx,organ medium treated; Tx,thymus grafted and untreated intact rats at 7, 8 and $17^2/7$ weeks, Reconstitution Experiment II.

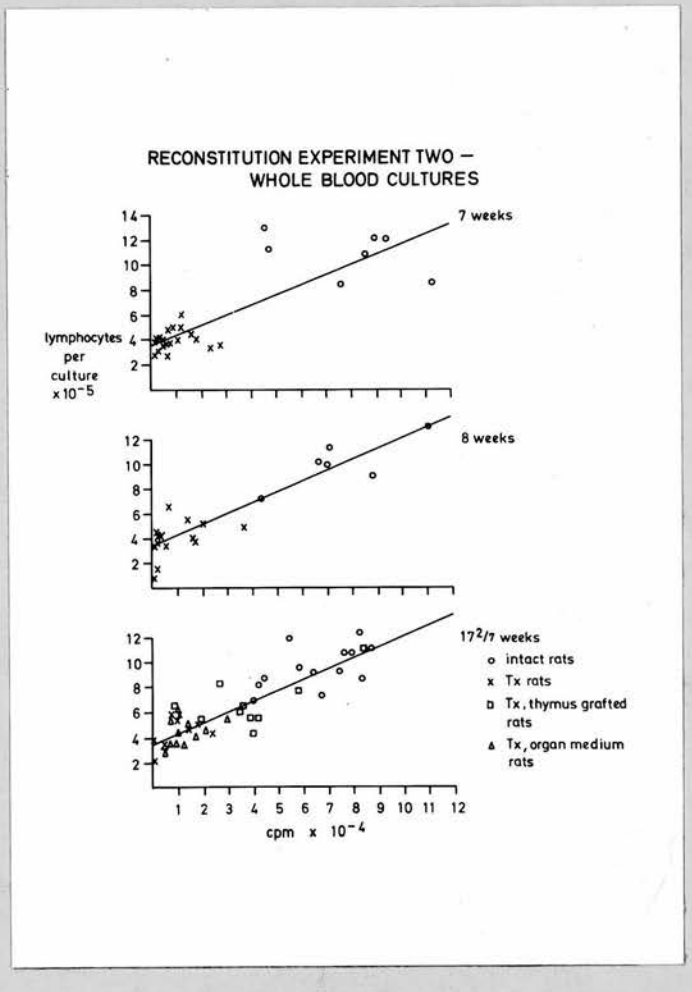


Figure 4.12 Plots of lymphocyte number and isotope incorporation in PHA stimulated whole blood samples from intact, thymectomized, thymectomized-thymus grafted and thymectomized-organ medium treated rats at 7, 8 and $17^2/7$ weeks. Reconstitution Experiment II.

Table 4.3 Blood picture of groups of Intact, Tx, thymus grafted, Tx, organ medium treated, and Tx, untreated rats at 17 2/7 weeks of age. Reconstitution Experiment II.

Rats (number)	mean ± S.D.		% Lym.	Mean ± SD	% Poly.	Mean ± SD	% Mono
	Total WBC /mm^3.	Total Lym. /mm^3.		Total Poly. /mm^3.		Total Mono. /mm^3.	
Intact (13)	10,194 ± 1,774	9,549 ± 1,690	93.7	574 ± 220	5.6	71 ± 51	0.7
Tx, thymus-grafted (10)	7,292 ± 1,932	6,696 ± 1,936	91.8	537 ± 257	7.4	59 ± 32	0.8
Tx, organ medium (12)	5,003 ± 1,096	4,255 ± 1,025	85.0	690 ± 311	13.8	58 ± 72	1.2
Tx, untreated (10)	5,392 ± 1,411	4,516 ± 1,163	83.7	796 ± 365	14.8	80 ± 33	1.5

Table 4.4 Results from linear regression analysis of the data in figure 4.12, in which the lymphocyte number and PHA response of individual blood samples over the three cultures are compared.

Age of rats at culture. (weeks)	Mean lymphocyte count of intact rats. per 0.1 ml. $(\times 10^{-2})$	Mean number of unresponsive lymphocytes per culture. $(\times 10^{-2})$	Mean percentage unresponsive cells in blood of intact rats.	Gradient $(\times 10^{-1})$	Coeffieient of linear regression (r)
7	10,922	3,588	32.9	0.8037	0.8300
8	10,107	3,346	33.1	0.8664	0.9129
$17\ ^{2}/7$	9,549	3,571	37.4	0.8510	0.8725

DISCUSSION

The experiments described in this chapter have confirmed that, in the rat, thymectomy performed within 48 hours after birth impairs the development of the circulating pool of PHA-responsive cells. The transplantation, at 50 or 65 days of age, of two syngeneic thymuses from neonatal donors, either subcutaneously or beneath the kidney capsule was found to partially restore the PHA response of thymectomized rats within four weeks following transplantation.

Linear regression analysis of the lymphocyte counts and the isotope uptake in PHA-stimulated blood samples suggested that the PHA response was a good measure of the number of circulating T cells. Such analysis produced a value, of 30 - 40% for the number of unresponsive cells (Bcells) in the whole blood of intact rats. This result was related to that reported by Balch and Feldman who found that 50 - 60% of rat peripheral blood lymphocytes stained with a heterologous anti-T-cell serum (Balch, C.M. and Feldman, J.D. 1974). Similar findings have been reported using anti-T-cell serum in the mouse (Raff, M.C. 1971). For human blood, Nowell found that 60% of the lymphocytes were capable of responding to PHA or forming rosettes with sheep erythrocytes (Nowell, P.C. et al 1975).

The variation in the PHA response of untreated animals and the low, and not baseline, response of thymectomized rats to PHA made necessary the use of quite large numbers of rats in each experimental group. Although the assay detected the increase in PHA response brought about by thymus grafting it was not known whether the assay was sensitive enough to demonstrate cases of less complete immunological restoration. The lack of restoration in the organ medium-treated group might be explained in this way, although the very close similarity between this group and the untreated thymectomized rat group made this possibility seem unlikely. Other

explanations for the failure to observe restoration in the thymus organ medium treated group include: not enough thymic replacement factor injected. 40 injections, over eight weeks, was considered to be quite a lengthy period of treatment and it is doubtful that a longer period or greater volume injected would have been more successful. No thymic factor was secreted or synthesized by the explants. This question is considered more fully in chapter six, where evidence concerning the viability of the thymus explants is presented. In the thymus organ cultures carried out for Reconstitution Experiment II, culture medium containing syngeneic serum was used. In the in vitro studies described in chapter six, tissue culture medium supplemented with FCS was normally used. Syngeneic serum was used in Reconstitution Experiment II to avoid having to set up another experimental group, that of rats injected with control (uncultured) medium, as the foreign antigens in heterologous serum might have affected the immune response. A possible drawback in the use of syngeneic serum was that epithelial outgrowths (epithelial cells are thought to be responsible for thymic humoral factor production) in thymus explants were reported to be more prevalent in cultures done in medium containing foreign antigens (Van den Tweel, J. 1971). Another possible explanation is that the treated rats were too old for restoration by purely humoral factors. There is evidence that when the age of neonatally thymectomized mice exceeds about 50 days they become refractory to treatment with thymus humoral factors. This is thought to be due to the loss of a post-thymic immunoincompetent cell population (Stutman, O, 1969 c) (Stutman, O, 1975). No similar studies have been reported for the rat. The treatment of the organ medium group was started when the rats were 65 days of age to allow comparisons of the PHA response of mature rats both before and after treatment. It is not known how successful an earlier schedule of thymus organ culture medium injections

would have been in bringing about increased PHA responses.

One reason why so few thymus grafts were recovered at autopsy was probably the difficulty in locating the grafts. This would explain why more grafts were recovered in the second experiment in which the grafts were placed under the kidney capsule. There was little correlation between increased PHA response and the recovery of the thymus graft. Little difference was found in the degree of the restoration of the PHA response when the thymus graft was placed subcutaneously or under the kidney capsule.

In summary, the thymic dependence of PHA transformation has been confirmed using a whole blood culture technique. Partial restoration of the PHA response and lymphocyte count in thymectomized rats has been demonstrated following syngeneic thymus transplantation. No restoration of the above parameters was observed following treatment of a group of thymectomized rats with a syngeneic preparation of cell-free medium obtained from thymus organ cultures. The whole blood-PHA assay was thought to give a good measure of the peripheral blood T cell population. The usefulness of the assay in measuring reconstitution by thymic factor preparations was limited by the need for quite large numbers of experimental animals and large volumes of thymic humoral factors.

CHAPTER FIVE

TISSUE EXTRACT STUDIES

OBJECTS AND INTRODUCTION

The work described in this chapter was carried out in order to investigate whether incubation of lymphoid cells with thymic extracts might lead to increased responses to PHA and whether such an effect could be used as the basis for an in vitro assay for the 'thymic humoral factor'.

The ability to respond to PHA is a property of a fairly mature T lymphocyte and therefore the target cell for such an effect of a thymic factor must be an immature T lymphocyte or a prethymocyte. Lymphocytes from three sources - the spleen, bone marrow and thymus were studied. These organs were thought to contain less mature cells than those found in the peripheral blood and lymph nodes. As intact rat spleen cells are highly responsive to PHA (see results of chapters two and three), thymectomized rats were used as donors of the spleen cells. Neonatal thymectomy had previously been shown to decrease the response of spleen cells to PHA (see experiment (3A), chapter three). The poorer response to PHA of spleen cells from thymectomized donors compared to the response obtained from intact rat spleen cells meant that relatively small increases in PHA responsiveness should be detected more easily because of a better "signal to noise ratio".

METHODS

I. Preparation of tissue extracts.

Thymus and spleen extracts were prepared from rat tissues using procedures very similar to those described by Allan Goldstein for the preparation of Thymosin (Goldstein, A.L. et al 1970 b). The same nomenclature concerning the purity of the extract i.e. fraction one (F_1), fraction two (F_{II}), etc as used by Goldstein was used in this study. Three batches of extract were used in the lymphocyte cultures described in this chapter - batches 3, 4 and 5. The details of the rats used to prepare these batches of extract and other relevant information are shown in table 5.1.

The rats were killed by ether anaesthesia and, within about two minutes after death, the thymus and/or spleen was carefully dissected out and placed on ice in a sterile plastic bijou. Sterile instruments and aseptic technique were used. The tissue was stored at -20°C until further treatment.

The frozen organs were allowed to thaw at room temperature and any obvious blood vessels removed. The tissue was then washed several times with sterile physiological saline and the tissue wet weight obtained. The tissue was then homogenised, using a Silverson heavy duty laboratory mixer emulsifier, in physiological saline, tissue wet weight: saline, 1:3 (batches 3 and 4); tissue wet weight: saline, 2:3 (batch 5). The homogenation was carried out in the cold room with the homogeniser head immersed in an ice bath. The tissue was homogenised during two 2½ minute runs at maximum speed with a 1 minute rest between runs. The homogenate was then placed into sterile glass centrifuge tubes and centrifuged at 3,000 rpm in an MSE Minor centrifuge. The supernatant consisted of about 60% of the total volume (batches 3 and 4) and was pipetted out of the tubes. The supernatant of batch 5 was so small, about 20%, that a further volume of saline was added and the homogenate recentrifuged and a further supernatant obtained. The low speed supernatant was divided between pairs of sterile 25 ml polycarbonate MSE centrifuge tubes and was centrifuged for one hour, under refrigeration, at 40,000 rpm in an MSE superspeed 50 centrifuge. The resulting supernatant was then removed and either stored in aliquots, designated fraction one, or subjected to the heat treatment.

The heat treatment entailed placing small volumes (1 - 6 ml) of extract fraction one into sterile glass bijous or universal containers and putting these into a water bath at 74 - 78°C for 20 minutes, and then cooling the extract on ice. This treatment caused a large

precipitate to form, especially in the case of batch 5 where a small volume of saline was added to facilitate removing the extract from the glass vessels. The batch 5 extract was given a preliminary centrifugation at 3,000 rpm for 15 minutes and then the heated extract was centrifuged at 40,000 rpm for one hour. The high speed supernatant, designated fraction two, was either aliquotted and stored in the deep freeze (batch 4) or stored in bulk in the deep freeze (batch 5).

Only one batch of extract, batch 5, was purified to the fraction three stage. This involved the slow addition of F_{II} extract to acetone that had been cooled to about -30°C by the addition of dry ice. 35 ml. of spleen F_{II} was added to 300 ml. acetone and 30 ml. of thymus F_{II} was added to 250 ml. acetone. The precipitates were filtered under pressure using a side-arm flask and a sinter glass filter funnel. Each precipitate was washed with about 40 ml. of cold acetone, scraped off the filter, placed in a plastic bijou and dried overnight in a vacuum desiccator. The solid was then extracted with phosphate buffered saline* (PBS), pH 7.2. 15 ml. of PBS was shaken with each acetone precipitate for 10 minutes. The solution was then centrifuged at 3,000 rpm for 5 minutes and the supernatant stored in the deep freeze. 5 ml. of PBS was then added to the remaining, undissolved precipitate and this was left at 4°C overnight. During the following morning the 5 ml. solution was removed from the white-coloured residue by centrifugation at 3,000 rpm for 5 minutes. This solution was added to the 15 ml. obtained on the previous day. The extract, called fraction three, was then sterilized by passage through a millipore filter of pore size 0.45μ, aliquotted into 1 ml. volumes and stored at -20°C.

* Phosphate buffered saline:

sodium chloride, 8.5 g.
disodium hydrogen phosphate, 1.5 g.
potassium dihydrogen phosphate, 0.43 g.
} 1 litre distilled water.

The F_{III} thymic extract was a very pale brown colour and the F_{III} splenic extract was a shade darker. The F_{II} extracts were lighter coloured than the F_{I} extracts. The splenic F_{I} extract was the most darkly coloured - red-brown.

Table 5.1 Details of the preparation of thymic and splenic extract, batches 3, 4 and 5

Extract batch	Tissue	Rats used (sex)	Age of rats (days)	Date rats killed	Tissue wet weight (g)	Extract fraction F_I date prepared	F_{II}	F_{III}
3	thymus	20 OLAC*, male female	100 - 104	23.5.74	10	31.7.74	-	-
	spleen				12			
4	thymus	10 ADRI, male	41	31.10.74	3	31.10.74	31.10.74	-
5	thymus	13 ADRI, male	54	13.1.75	43	12.3.75	18.3.75	18.4.75
		76 OLAC, male female	54 - 80	24.2.75				
	spleen	13 ADRI, male	54	13.1.75	58	12.3.75	18.3.75	17.4.75
		76 OLAC, male female	54 - 80	24.2.75				

* OLAC - (A random bred strain of Sprague-Dawley rats obtained from Oxford laboratories and bred within the department of Clinical Surgery).

II. Protein Assay

As the active moiety in most thymic extract preparations has been ascribed to protein or peptide fractions, quantitation of the extract on a total protein basis was considered as that most appropriate for this study. The method of protein assay used was a modification of that devised by Lowry and co-workers (Lowry, O.H. et al 1951).

Reagents.

Reagent A: 2% sodium carbonate in 0.1 M sodium hydroxide.

Reagent B: part I - 1% copper sulphate.

part II - 2% sodium or potassium tartrate.

On the day of the assay equal volumes of parts I and II were mixed and allowed to stand for 30 minutes before use.

Reagent C: 50 ml. of Reagent A was mixed with 1 ml. of Reagent B This was discarded after one day.

Reagent D: Folin and Ciocalteu Phenol reagent (BDH Chemicals Ltd.) This was diluted, 1 ml. plus 19 ml. distilled water.

Protein standards.

1 g. of bovine serum albumin (BSA), fraction 5 from bovine plasma (Armour Pharmaceutical Company Ltd., Eastbourne, England) was dissolved to make a 1% solution with distilled water. This was then diluted to make standard concentrations between 100 and 1000 μg. per ml.

Experimental procedure.

(1) A series of tubes were set up containing 0.2 ml. of the standard or test solution.

(2) 1 ml. of Reagent C was added to each tube and the contents were well mixed and allowed to stand for 10 minutes.

(3) 1 ml. of Reagent D was added rapidly and the contents were immediately and thoroughly mixed.

(4) After a minimum of 30 minutes the samples were read in a Unicam Sp 600, series 2 spectrophotometer set at wavelength 750 mμ .

Protein determinations were carried out on each extract fraction after the extract had been aliquotted and stored in the deep freeze. The protein concentrations of the various fractions of extract batches 3, 4 and 5 are shown in table 5.2.

Table 5.2 Protein concentration of extract batches 3, 4 and 5.

Extract batch	Tissue	Extract fraction		
		F_I	F_{II}	F_{III}
		protein concentration mg/ml		
3	thymus	17.9	-	-
	spleen	28.9	-	-
4	thymus	9.1	2.3	-
5	thymus	12.2	2.9	1.8
	spleen	26.3	3.8	3.0

III. Lymphocyte culture methods.

The culture methods used in this chapter were essentially those described in chapter two. When different techniques were used these are described in the section on experimental design and results.

Tritiated thymidine of low specific activity (5 Ci/m.mol.) was used to assess the response to PHA in experiments (6) (7) (8) and (9). In all the other experiments thymidine (21 - 27 Ci/m.mol.) was used.

EXPERIMENTAL DESIGN AND RESULTS.

Experiments:

(1) The effect of an 8 hour preincubation and continuous culture with thymic or splenic extract batch 3, fraction I, on the incorporation of tritiated thymidine in PHA -stimulated and unstimulated intact rat bone marrow cells.

(2) The effect of an 8 hour preincubation and continuous culture with thymic extract batch 3, fraction I, on the incorporation of tritiated thymidine in PHA-stimulated and unstimulated thymectomized rat bone marrow and spleen cells.

(3) The effect of a 24 hour preincubation and continuous culture with thymic extract batch 3, fraction I, on the incorporation of tritiated thymidine in PHA-stimulated and unstimulated thymectomized rat bone marrow cells.

(4) The effect of a 10 hour preincubation and continuous culture with thymic extract batch 4, fractions I and II, on tritiated thymidine incorporation in PHA-stimulated and unstimulated thymectomized rat spleen cells.

(5) The effect of (A) a 9 hour preincubation with thymic extract batch 4, fractions I and II, followed by culture in "extract-free" medium, and (B) a 9 hour preincubation and continuous culture with thymic extract batch 4, fractions I and II, on tritiated thymidine incorporation in PHA-stimulated and unstimulated thymectomized rat spleen cells.

(6) The effect of a 16 hour preincubation and continuous culture with thymic or splenic extract batch 5, fraction III, on the

incorporation of tritiated thymidine in PHA-stimulated and unstimulated thymectomized rat spleen cells.

(7) The effect of a 16 hour preincubation with thymic or splenic extract batch 5, fraction III, followed by culture in "extract-free" medium, on the incorporation of tritiated thymidine in PHA-stimulated and unstimulated thymectomized rat spleen cells.

(8) The effect of a 16 hour preincubation with thymic or splenic extract batch 5, fraction III, followed by culture in "extract-free" medium, on the incorporation of tritiated thymidine in PHA-stimulated and unstimulated intact rat bone marrow cells.

(9) The effect of a 16 hour preincubation with thymic or splenic extract batch 5, fraction III, followed by culture in "extract-free" medium, on the incorporation of tritiated thymidine in PHA-stimulated and unstimulated rat thymus cells.

Experiment (1) The effect of an 8 hour preincubation and continuous culture with thymic or splenic extract batch 3, fraction I, on the incorporation of tritiated thymidine in PHA-stimulated and unstimulated intact rat bone marrow cells.

Culture vials were set up to contain 1 ml. of medium 199 supplemented with 10 mM HEPES and 10% FCS. A range of between 0 and 450 μg. of thymic or splenic extract batch 3, F_I, diluted in medium 199 was then added in 100 μl. volumes. 2.8×10^6 bone marrow cells, prepared from two intact male rats aged 64 days were then added. This was followed by the addition of a thymectomized rat erythrocyte preparation. The cells were then incubated at 37°C for 8 hours before 2.5 μl. of PHA in 100 μl. saline or 100 μl saline was added. After incubation for a further 48 hours, 1 μCi. of tritiated thymidine was added and the culture was terminated 22 hours later.

The results of this culture are shown in table 5.3. No significantly increased PHA responses were seen at any concentration of extract. In the case of the splenic extract, increased isotope incorporation in cultures containing PHA was matched with increased isotope incorporation in the absence of PHA.

Table 5.3 Isotope incorporation in cultures of (2.8×10^6) intact rat bone marrow cells following an 8 hour preincubation and continuous culture with thymic and splenic extract batch 3, fraction I.

Extract batch 3 F_I µg./culture.	Thymic extract			Splenic extract		
	2.5 µl. PHA	0 µl. PHA	SI	2.5 µl. PHA	0 µl. PHA	SI
	cpm, mean ± S.D.			cpm, mean ± S.D.		
0	15,686 ± 996	11,843 ± 2,654	1.3	15,686 ± 996	11,843 ± 2,654	1.3
14	18,444 ± 1,357	11,964 ± 1,664	1.5	24,311 ± 1,135	14,055 ± 1,599	1.7
28	15,312 ± 1,653	11,040 ± 3,776	1.4	23,723 ± 813	14,727 ± 2,322	1.6
56	17,288 ± 953	9,695 ± 4,475	1.8	21,789 ± 1,001	17,304 ± 995	1.3
112	15,638 ± 1,000	7,240 ± 1,365	2.2	22,399 ± 1,874	14,414 ± 681	1.6
225	17,736 ± 805	7,393 ± 4,357	2.4	14,116 ± 599	9,584 ± 2,682	1.5
450	15,675 ± 2,015	7,623 ± 234	2.1	11,999 ± 1,006	5,688 ± 667	2.1

Experiment (2) The effect of an 8 hour preincubation and continuous culture with thymic extract batch 3, fraction I, on the incorporation of tritiated thymidine in PHA-stimulated and unstimulated rat bone marrow and spleen cells.

A range of between 0 - 900 µg. of thymic extract batch 3, fraction one, was added to culture vials containing 1 ml. medium 199 supplemented with 10 mM HEPES and 10% FCS. 2.5×10^6 bone marrow cells or 1.3×10^6 spleen cells, prepared from the same 70 day old thymectomized male rat were then added to designated vials. The cells were incubated at 37°C for 8 hours and then 2.5 µl. of PHA in 100 µl. saline or 100 µl. saline and 100 µl of a thymectomized rat erythrocyte preparation were added. After a further 49 hours culture, 1 µCi. of tritiated thymidine was added, and the culture was terminated after a pulse of 18 hours.

The results are shown in table 5.4. The presence of the thymic extract made little difference to isotope incorporation in both spleen and bone marrow cell cultures. A slight increase in the PHA response of both spleen and bone marrow cell cultures was seen in the presence of 56 µg. thymic extract. No increased responses were seen at any other extract concentration. At the highest concentration of extract the isotope incorporation tended to be reduced.

Table 5.4. Isotope incorporation in cultures of 2.5×10^6 thymectomized rat bone marrow cells and 1.3×10^6 thymectomized rat spleen cells following an 8 hour preincubation and continuous culture with thymic extract batch 3, fraction I.

Cells (number)	Thymic extract Batch 3, F_I. μg./culture	2.5 μl. PHA	0 μl. PHA	SI
		cpm, mean ± S.D.		
Tx rat Bone marrow cells (2.5×10^6)	0	13,283 ± 1,465	10,631 ± 358	1.2
	14	13,064 ± 960	11,349 ± 374	1.2
	28	12,731 ± 876	11,162 ± 297	1.1
	56	15,278 ± 962	10,467 ± 1,455	1.4
	112	13,115 ± 2,258	10,723 ± 451	1.2
	450	11,071 ± 760	11,301 ± 759	1.0
	900	9,241 ± 398	12,218 ± 1,096	0.8
Tx rat Spleen cells (1.3×10^6)	0	320 ± 23	291 ± 42	1.1
	14	314 ± 14	283 ± 95	1.1
	28	309 ± 14	311 ± 74	1.0
	56	481 ± 241	296 ± 143	1.6
	112	304 ± 74	302 ± 28	1.0
	450	300 ± 80	230 ± 76	1.3
	900	125 ± 11	161 ± 89	0.8

Experiment (3) The effect of a 24 hour preincubation and continuous culture with thymic extract batch 3, fraction I, on the incorporation of tritiated thymidine in PHA-stimulated and unstimulated rat bone marrow cells.

As it was possible that an 8 hour preincubation was too short a period in which to effect an increased responsiveness to PHA, a 24 hour preincubation was used in this experiment. Another variable, the time of addition of the erythrocytes, was studied. Thus the erythrocytes were added at the same time as the extract or 24 hours after the extract when PHA or saline was added. Following the suggestive result of experiment (2), a thymic extract concentration of 60 μg. per culture was chosen, along with control cultures not containing extract, for a PHA dose response study. The culture was set up, exactly as in the previous two experiments, using medium 199 supplemented with 10 mM HEPES and 10% FCS. 2.6×10^6 bone marrow cells from an 84 day old neonatally thymectomized rat were cultured for a total of 93 hours, PHA was present for the last 69 hours. The response to PHA was assessed by a pulse of 20 hours.

The results from this study are shown in tables 5.5 and 5.6 and 5.7 and figure 5.1. The increased isotope incorporation in cultures containing extract seemed to be due to an effect on the unstimulated cells, see table 5.5 and figure 5.1. In these cultures the erythrocytes were added at the onset of the culture. Little difference in isotope incorporation was found, however, between cultures in which the erythrocytes were added at the onset or after 24 hours, table 5.6. No sign of an increased PHA response was found in the presence of 60 μg. thymic extract over the range of PHA concentrations studied, see table 5.7 and figure 5.1. Maximum isotope incorporation was found at the PHA concentration of 4 μl. per culture, good stimulation also occurred at 2.5 μl. PHA per culture.

Table 5.5 Isotope incorporation in cultures of 2.6×10^6 thymectomized rat bone marrow cells following a 24 hour preincubation and continuous culture with thymic extract, batch 3, fraction I.

Thymic extract, batch 3, F_I. µg./culture	2.5 µl. PHA	0 µl. PHA	SI
	cpm., mean ± SD		
0	5,576 ± 725	3,621 ± 803	1.5
30	7,384 ± 770	6,558 ± 395	1.1
60	6,560 ± 693	5,706 ± 472	1.1
90	6,282 ± 896	5,919	1.1

Table 5.6 The effect on isotope incorporation of varying the time of addition of erythrocytes to cultures of 2.6×10^6 thymectomized rat bone marrow cells containing either 0 or 60 µg. thymic extract batch 3, fraction I.

Thymic extract, batch 3, F_I. µg./culture	Time of addition of erythrocytes (hours).	2.5 µl. PHA	0 µl. PHA	SI
		cpm., mean ± SD		
0	0	5,576 ± 725	3,621 ± 803	1.5
	24	5,456 ± 423	5,196 ± 305	1.1
60	0	6,560 ± 693	5,706 ± 472	1.2
	24	6,768 ± 723	4,531 ± 494	1.5

Table 5.7 Isotope incorporation in cultures of 2.6×10^6 thymectomized rat bone marrow cells following a 24 hour preincubation and continuous culture with either 0 or 60 µg. thymic extract batch 3, fraction I, over a range of PHA concentrations.

PHA µl./culture.	Thymic extract batch 3, F_I. (µg./culture).			
	0 µg.		60 µg.	
	cpm, mean ± S.D.	SI	cpm, mean ± S.D.	SI
0	3,621 ± 808	-	5,706	-
1.25	3,817 ± 948	1.1	6,133 ± 724	1.1
2.5	5,576 ± 725	1.5	6,560 ± 693	1.1
4.0	6,245 ± 514	1.7	7,320 ± 1,453	1.3
8.0	4,359 ± 266	1.2	6,237 ± 1,494	1.1

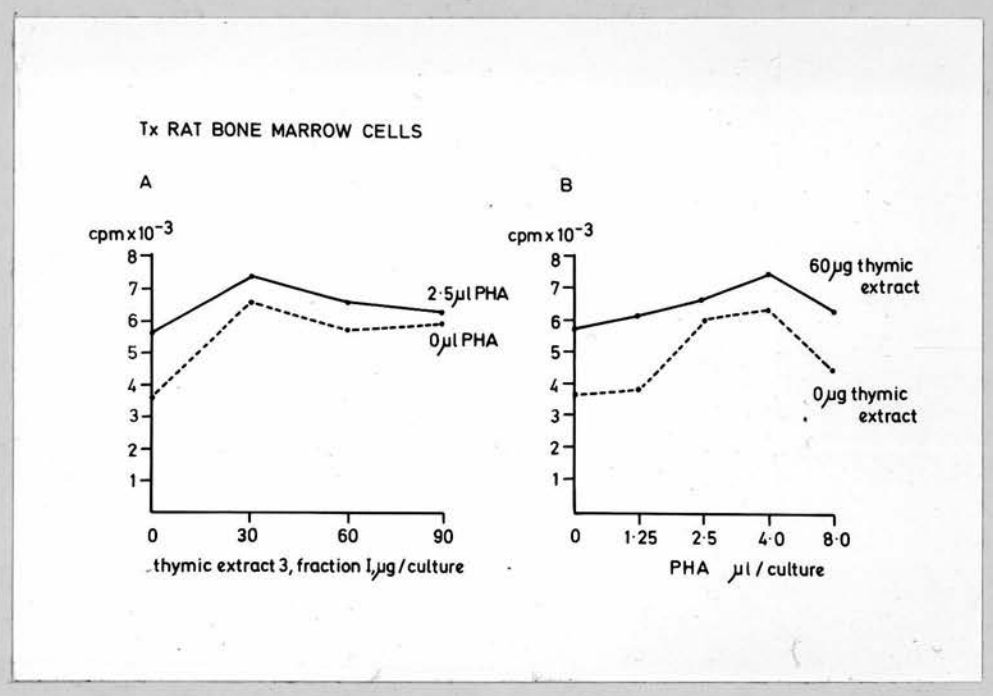


Figure 5.1 Isotope incorporation in PHA-stimulated and unstimulated cultures of 2.6×10^6 thymectomized rat bone marrow cells following a 24 hour pre-incubation and continuous culture with thymic extract batch 3, fraction I.

Experiment (4) <u>The effect of a 10 hour preincubation and continuous culture with thymic extract batch 4, fractions I and II, on tritiated thymidine incorporation in PHA-stimulated and unstimulated rat spleen cells.</u>

A range of between 0 and 215 μg. of thymic extract batch 4, fractions I and II, was added to culture vials containing 1 ml. medium 199 supplemented with 10 mM HEPES and 10% FCS. 2.4×10^6 spleen cells from a thymectomized male rat aged 110 days were then added to the vials. The cells were incubated for 10 hours at 37°C before 2.5 μl. PHA or saline and 100 μl. of a thymectomized rat erythrocyte preparation were added. After a further 38 hours, tritiated thymidine was added and the culture was terminated 23 hours later.

The results are presented in table 5.8 and figure 5.2. Both fractions of thymic extract were found to depress the isotope uptake in both PHA-stimulated and unstimulated spleen cells at all extract concentrations tested. The suppressive effect of the F_{II} extract was consistently greater than that of the F_I and the suppressive effect of both fractions increased with increasing concentrations of extract.

Table 5.8 Isotope incorporation in cultures of 2.4×10^6 thymectomized rat spleen cells following a 10 hour preincubation and continuous culture with thymic extract batch 4, fractions I and II.

Thymic extract batch 4. µg./culture.	Fraction I.			Fraction II		
	2.5 µl. PHA	0 µl. PHA	SI	2.5 µl. PHA	0 µl. PHA	SI
	cpm, mean ± S.D.			cpm, mean ± S.D.		
0	5,272 ± 415	677 ± 42	7.8	5,272 ± 415	677 ± 42	7.8
27	4,322 ± 411	551 ± 163	7.8	2,550 ± 69	380 ± 148	6.7
54	4,171 ± 588	375 ± 132	11.1	1,729 ± 194	191 ± 58	9.1
107	4,076 ± 789	372 ± 72	11.0	1,276 ± 242	142 ± 107	9.0
215	2,643 ± 497	296 ± 24	8.9	193 ± 30	103 ± 2	1.9

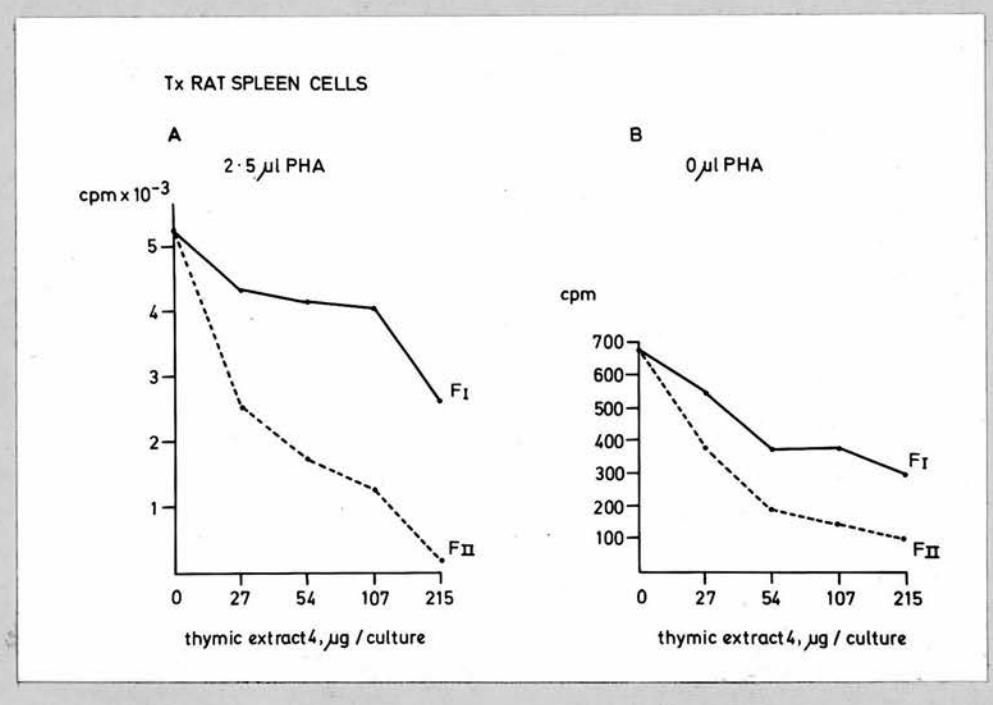


Figure 5.2 Isotope incorporation in (A) PHA-stimulated, and (B) unstimulated cultures of 2.4×10^6 thymectomized rat spleen cells following a 10 hour preincubation and continuous culture with thymic extract batch 4, fractions I and II.

Experiment (5) <u>The effect of (A) a 9 hour preincubation with thymic extract batch 4, fractions I and II, followed by culture in "extract-free" medium, and (B) a 9 hour preincubation and continuous culture with thymic extract batch 4, fractions I and II, on tritiated thymidine incorporation in PHA-stimulated and unstimulated thymectomized rat spleen cells.</u>

The negative or equivocal results of the previous experiments and the finding that the F_{II} thymic extract strongly suppressed tritiated thymidine incorporation led to the question of whether PHA responsiveness was increased, but not apparent, because of the presence throughout the response to PHA phase of the thymic extract. In order to answer this question the culture system was modified by carrying out the preculture in plastic universal containers (UC's), containing six times the standard culture volume. At the end of the preincubation period the cells were centrifuged and resuspended in the same volume of medium without tissue extract.

Each UC contained 7.2 ml. of medium 199 supplemented with 10 mM HEPES and 10% FCS. Also contained in the 7.2 ml. was 15×10^6 spleen cells from a thymectomized rat aged 72 days and thymic extract F_I or F_{II} either 642 μg. or 162 μg. Control cultures without tissue extract were also carried out. The UC's were placed, loosely-capped, into a sealable polythene box at an angle of approximately 30° to the horizontal. The box was then sealed, gassed with 5% CO_2 in air and placed into an incubator. After 9 hours incubation at 37°C the cells in each UC were pelleted by centrifugation at 2,000 rpm for 10 minutes. 7 ml. of medium was then carefully and accurately withdrawn from those vials designated "preculture to fresh" and replaced with 7 ml. unused medium not containing

extract. The cells were then resuspended by gentle agitation. From each UC, three 1 ml. aliquots were placed into standard 5 ml. plastic culture vials containing 4 µl. PHA in 100 µl. saline and three aliquots placed into vials containing 100 µl. saline. Each 1 ml. aliquot thus contained approximately 2.1 x 10^6 spleen cells and either 0, 22.5 or 90 µg. thymic extract. After a further 15 hours in culture at 37°C., 100 µl. of a thymectomized rat erythrocyte preparation was added to each vial. 38 hours after the addition of the spleen cells to the PHA, 1 µCi. of tritiated thymidine was added to each vial and the culture was terminated after a pulse of 23 hours.

The results from this experiment are shown in table 5.9. As individual cultures using the "preculture in bulk" method were smaller, 1.2 m. (1.0 ml. medium, cells and extract, 0.1 ml. PHA, 0.1 ml. erythrocytes) the amounts of extract per culture are not directly comparable to those in previous cultures. An increased PHA response was found in cells precultured in the lower concentration of both F_I and F_{II} thymic extract and then cultured in "extract-free" medium. This effect was not seen using the higher concentration of extract. When the extract was present throughout the culture the F_{II} thymic extract was again found to suppress isotope incorporation to a greater extent than the F_I extract. When cells were transferred to fresh medium after the 9 hour preincubation both PHA-stimulated and unstimulated cells subsequently incorporated more isotope than those cells cultured in unchanged medium. That this occurred in cells cultured with the thymic extract was attributed partly to the suppressive effect of the extract. In cells cultured without extract a slight increase in isotope uptake was found in the PHA-stimulated cells and a large increase occurred in the unstimulated cells. This was perhaps due to the removal of cell debris and other factors that inhibit tritiated thymidine incorporation in cultured lymphocytes.

Table 5.9 Isotope incorporation in cultures of 2.1 x 10^6 thymectomized rat spleen cells following a 9 hour preculture with thymic extract batch 4, fractions I and II, followed by either culture in "extract-free" medium (preculture to fresh) or culture in unchanged medium (continuous culture).

Type of culture.	Thymic extract batch 4 µg./culture	Fraction I			Fraction II		
		4 µl. PHA	0 µl. PHA	SI	4 µl. PHA	0 µl. PHA	SI
		cpm, mean ± S.D.			cpm, mean ± S.D.		
"Preculture to fresh"	0	12,422 ± 308	12,345 ± 1,270	1.0	12,422 ± 308	12,345 ± 1,270	1.0
	22.5	14,023 ± 131	9,192 ± 1,984	1.5	15,820 ± 551	11,656 ± 760	1.4
	90	11,744 ± 316	11,766 ± 141	1.0	8,475 ± 628	8,013 ± 866	1.1
"continuous"	0	10,356 ± 2,099	4,594 ± 392	2.3	10,356 ± 2,099	4,594 ± 392	2.3
	22.5	5,844 ± 102	3,117 ± 330	1.9	4,427 ± 375	2,893 ± 609	1.5
	90	4,909 ± 463	3,708 ± 583	1.3	556 ± 68	564 ± 84	1.0

Experiment (6) The effect of a 16 hour preincubation and continuous culture with thymic or splenic extract batch 5, fraction III, on the incorporation of tritiated thymidine in PHA-stimulated and unstimulated thymectomized rat spleen cells.

Plastic universal containers were set up containing 6.6 ml. EMEM supplemented with 5% FCS. 16.2×10^6 spleen cells, prepared from a thymectomized rat aged 129 days and either 0, 180 or 720 µg. of thymic or splenic extract batch 5, fraction III, were present within the 6.6 ml. After a 16 hour incubation at 37°C, 1 ml. aliquots were placed into vials containing 4 µl. PHA in 100 µl. saline or 100 µl. saline and 100 µl. of a thymectomized rat erythrocyte preparation. After a further 48 hours culture, 1 µCi. of tritiated thymidine was added to each vial and the cells were harvested 24 hours later.

The results are shown in table 5.10. Both thymic and splenic extracts (fraction III) were found to strongly depress isotope uptake in both PHA-stimulated and unstimulated spleen cell cultures. The suppressive effect of the thymic extract was slightly greater than that of the splenic extract. The suppressive potency of the F_{III} extract was similar to that of the F_{II} extract, as judged from previous cultures.

Table 5.10 Isotope incorporation in cultures of 2.5×10^6 thymectomized rat spleen cells following a 16 hour preincubation and continuous culture with thymic and splenic extract batch 5, fraction III.

Type of culture.	Extract batch 5 F_{III} µg/culture.	Thymic extract			Splenic extract		
		4.0 µl. PHA	0 µl PHA	SI	4.0 µl. PHA	0 µl. PHA	SI
		cpm, mean ± S.D.			cpm, mean ± S.D.		
"continuous"	0	26,604	494 ± 14	54	26,604	494 ± 14	54
	27	18,187 ± 397	150 ± 14	121	22,754 ± 367	236 ± 49	96
	109	1,559 ± 93	65 ± 14	24	5,847 ± 405	78 ± 4	75

Experiment (7) <u>The effect of a 16 hour preincubation with thymic or splenic extract batch 5, fraction III, followed by culture in "extract-free" medium, on the incorporation of tritiated thymidine in PHA-stimulated and unstimulated thymectomized rat spleen cells</u>.

This was the first of three experiments: (7) (8) (9), in which thymectomized rat spleen cells, intact rat bone marrow cells and thymus cells respectively, were cultured with thymic and splenic extract batch 5, fraction III, using the "preculture to fresh" technique. Several alterations were made to the culture method used in experiment (5). The medium in experiments (7) (8) and (9) was EMEM supplemented with 5% FCS and not containing HEPES. Each universal container (UC) contained 6.6 ml. of medium, varying amounts of the thymic and splenic extract and the cells.

Spleen cells were prepared from two thymectomized male rats aged 144 days and 17.4 x 10^6 cells were added to each UC. The following amounts of thymic or splenic extract were present per 6.6 ml.: 0, 60, 120, 180, 360, 540 μg. This meant that after centrifugation and 1 ml. aliquots were taken, individual cultures contained approximately 2.6 x 10^6 spleen cells and respectively, 0, 9, 18, 27, 55, and 82 μg. of the thymic or splenic extract. The spleen cells were cultured with or without extract for 16 hours and then the UC's were centrifuged and, as described in experiment (5), the extract-containing medium was discarded and replaced with unused EMEM containing 5% FCS and no extract. 1 ml. aliquots were then made into vials containing either 4 μl. PHA in 100 μl. saline or 100 μl. saline, and 100 μl. of an erythrocyte preparation. The cells were then incubated for 45 hours at 37°C and pulsed with tritiated

thymidine for 24 hours prior to harvest.

The results are shown in table 5.11 and figure 5.3. All concentrations of thymic and splenic extract depressed isotope incorporation in both PHA-stimulated and unstimulated spleen cells. The thymic extract depressed isotope uptake in PHA-stimulated cells to a slightly greater extent than did the splenic extract. Both extracts were more effective in decreasing the isotope incorporation in unstimulated cells compared to cells responding to PHA.

Table 5.11 Isotope incorporation in cultures of 2.6×10^6 thymectomized rat spleen cells following a 16 hour preincubation with thymic or splenic extract batch 5, fraction III, and culture in "extract-free" medium.

Extract batch 5 F_{III} µg./culture	Thymic extract			Splenic extract		
	4 µl. PHA	0 µl. PHA	SI	4 µl. PHA	0 µl. PHA	SI
	cpm, mean ± S.D.			cpm, mean ± S.D.		
0	135,350 ± 9,103	5,694 ± 313	24	135,350 ± 9,103	5,694 ± 313	24
9	134,100 ± 4,994	2,818 ± 412	48	130,044 ± 17,389	2,631 ± 253	49
18	122,892 ± 7,718	1,965 ± 179	63	123,492 ± 3,612	2,323 ± 316	53
27	118,372 ± 1,327	1,506 ± 32	79	124,846 ± 4,367	1,859 ± 112	67
55	82,450 ± 4,197	641 ± 18	129	97,224 ± 4,606	900 ± 44	108
82	47,576 ± 3,555	545 ± 106	87	69,173 ± 8,562	603 ± 70	115

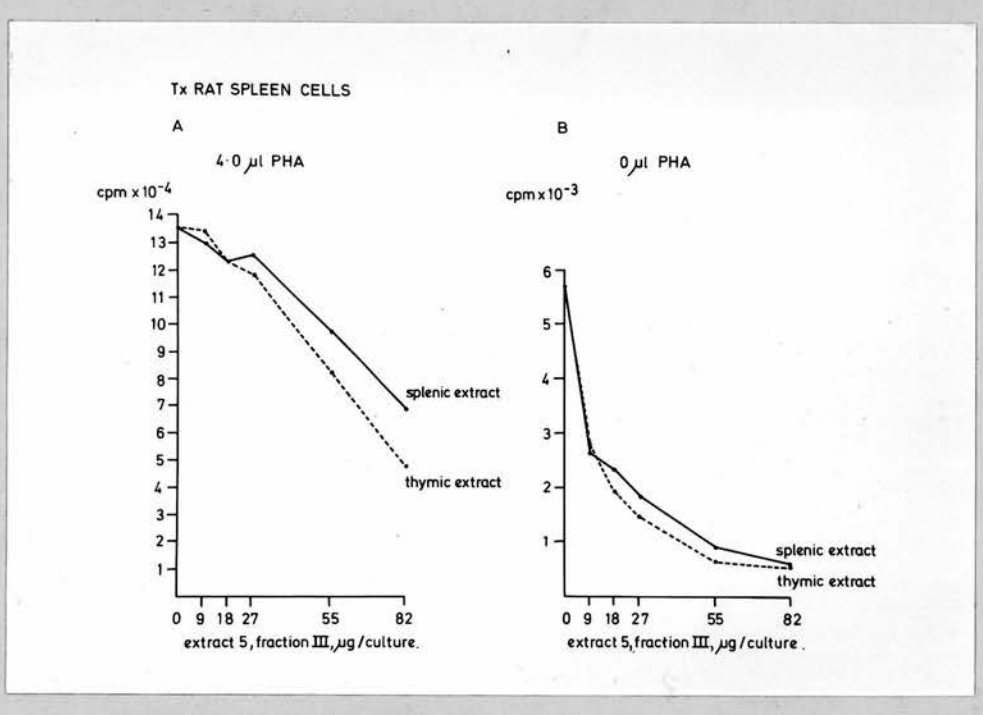


Figure 5.3 Isotope incorporation in (A) PHA-stimulated, and (B) unstimulated cultures of 2.6×10^6 thymectomized rat spleen cells following a 16 hour preincubation with thymic or splenic extract batch 5, fraction III, and culture in "extract-free" medium.

Experiment (8) The effect of a 16 hour preincubation with thymic or splenic extract batch 5, fraction III, followed by culture in "extract-free" medium, on the incorporation of tritiated thymidine in PHA-stimulated and unstimulated intact rat bone marrow cells.

This experiment was set up exactly as described in experiment (7). Bone marrow cells were prepared from two intact male rats, one aged 59 days and the other 73 days. 13.2×10^6 bone marrow cells were added to each UC along with the extract and EMEM. The cells were incubated with the extract for 16 hours and then centrifuged and aliquotted as previously described. Individual culture vials contained 2.0×10^6 bone marrow cells. After the cells had been cultured for 48 hours with PHA, tritiated thymidine was added and the cells were harvested after a further 19 hours.

The results are shown in table 5.12 and figure 5.4. Isotope incorporation was much lower than normal in both the PHA-stimulated and unstimulated bone marrow cell cultures. This was probably due to the lower than usual number of cells per culture. Slight increases in isotope uptake were found in both PHA-stimulated and unstimulated cells when precultured with the lower concentrations of both extracts, but particularly the thymic extract. Control cultures showed that the erythrocytes incorporated very little isotope, 14 and 13 cpm respectively in the presence and absence of PHA.

Table 5.12 Isotope incorporation in cultures of 2.0×10^6 intact rat bone marrow cells following a 16 hour preincubation with thymic or splenic extract batch 5, fraction III, and culture in "extract-free" medium.

Extract batch 5 F_{III} µg./culture.	Thymic extract			Splenic extract		
	4.0 µl. PHA	0 µl. PHA	SI	4.0 µl. PHA	0 µl. PHA	SI
	cpm, mean ± S.D.			cpm, mean ± S.D.		
0	568 ± 60	116 ± 21	4.9	568 ± 60	116 ± 21	4.9
9	702 ± 146	103 ± 12	6.8	636 ± 239	93 ± 20	6.8
18	766 ± 77	141 ± 42	5.4	525 ± 117	110 ± 2	4.8
27	593 ± 16	95 ± 4	6.2	474 ± 47	122 ± 36	3.9
55	609 ± 127	103 ± 1	5.9	486 ± 87	76 ± 12	6.4
82	530 ± 32	86 ± 10	6.2	455 ± 36	53 ± 8	8.6

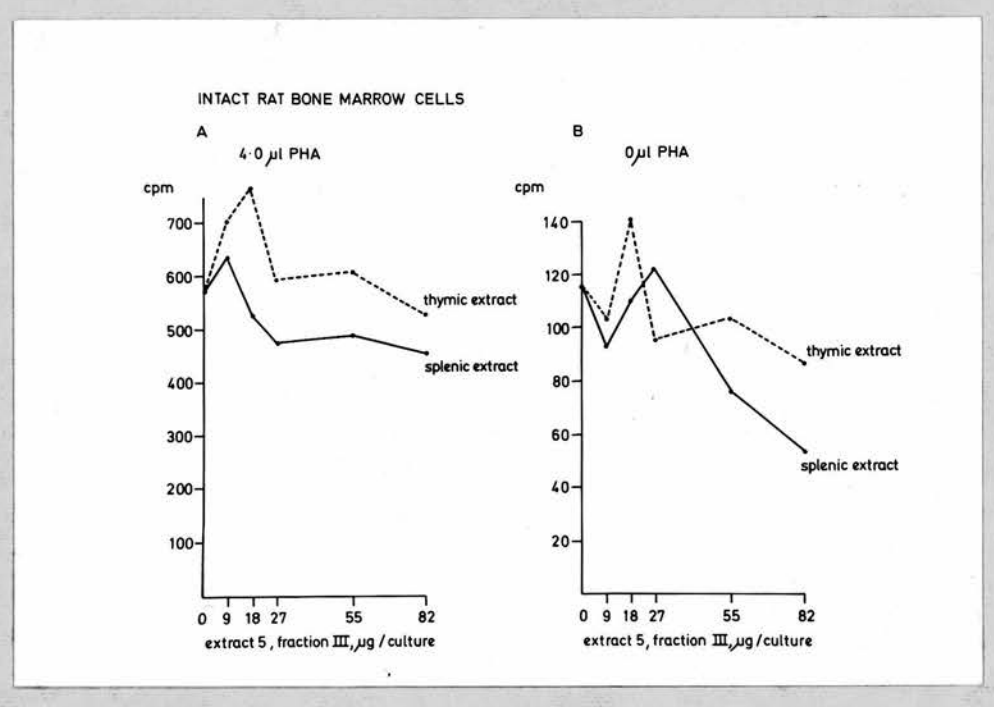


Figure 5.4 Isotope incorporation in (A) PHA-stimulated, and (B) unstimulated cultures of 2.0×10^6 intact rat bone marrow cells following a 16 hour preincubation with thymic or splenic extract batch 5, fraction III, and culture in "extract-free" medium.

Experiment 9) <u>The effect of a 16 hour preincubation with thymic or splenic extract batch 5, fraction III, followed by culture in "extract-free" medium, on the incorporation of tritiated thymidine, in PHA-stimulated and unstimulated rat thymus cells.</u>

20.2×10^6 thymus cells, prepared from a 61 day old rat, were precultured for 16 hours, with and without, various quantities of thymic and splenic extract as described in the previous two experiments. After resuspension in medium containing no extract and the addition to vials containing PHA or saline and erythrocytes, the thymus cells were cultured for a further 49 hours before tritiated thymidine was added and the cells harvested after a 24 hour pulse.

The results are shown in table 5.3 and figure 5.5. Increased responses to PHA were found in cells precultured in 9 and 18 μg. splenic extract and 27 μg. thymic extract. Increased isotope uptake was also found in unstimulated cells precultured with 18 μg. thymic extract and 9 and 18 μg. splenic extract. Reduced isotope incorporation was found in cells precultured in 55 and 82 μg. of extract. The responsiveness to PHA of the 3.1×10^6 thymus cells per culture was high compared with other thymus cell cultures (see tables 2.15 and 6.4). The lower serum concentration and greater cell number might account for the higher isotope incorporation found in this experiment. The possibility also exists that during the preparation of the cells some fragments of lymph node were included with the thymus.

Table 5.13 Isotope incorporation in cultures of 3.1 x 10^6 rat thymus cells following a 16 hour preincubation with thymic or splenic extract batch 5, fraction III, and culture in "extract-free" medium.

Extract batch 5 F_{III} µg./culture.	Thymic extract			Splenic extract		
	4.0 µl. PHA	0 µl. PHA	SI	4.0 µl. PHA	0 µl. PHA	SI
	cpm, mean ± S.D.			cpm, mean ± S.D.		
0	93,183 ± 4,273	662 ± 74	141	93,183 ± 4,273	662 ± 74	141
9	94,384 ± 10,285	781 ± 205	121	98,729 ± 3,975	906 ± 399	109
18	94,986 ± 2,190	903 ± 199	105	106,514 ± 5,583	871 ± 94	122
27	103,607 ± 8,593	752 ± 73	138	90,689 ± 4,483	827 ± 264	110
55	78,912 ± 8,593	534 ± 76	148	77,443 ± 15,859	649 ± 118	119
82	67,314 ± 2,392	378 ± 135	178	60,512 ± 2,281	382 ± 135	158

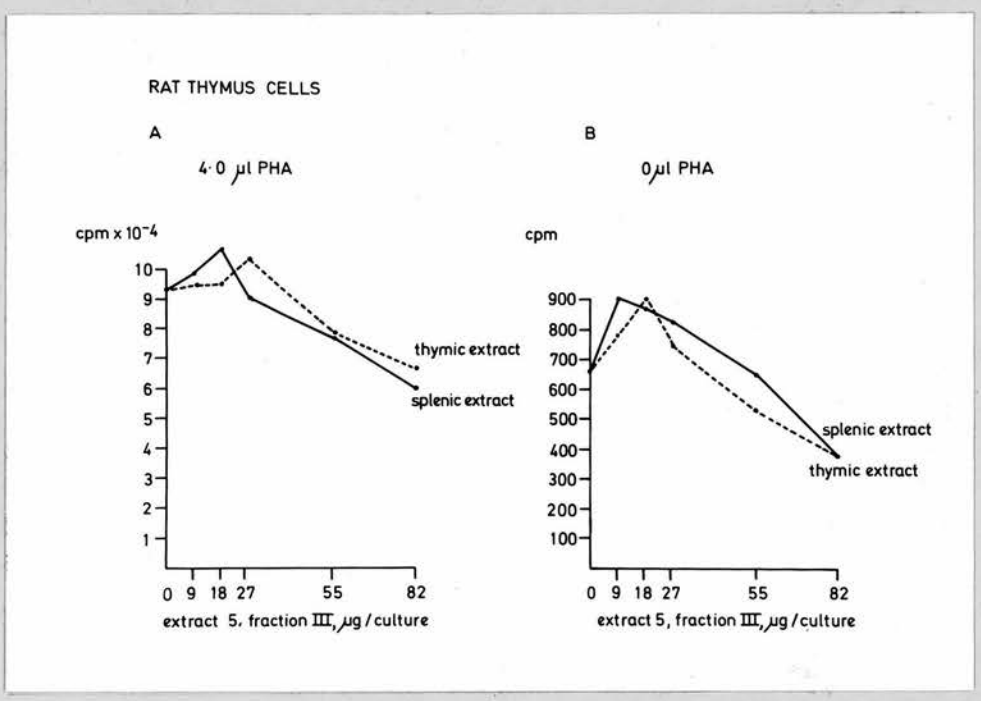


Figure 5.5 Isotope incorporation in (A) PHA-stimulated, and (B) unstimulated cultures of 3.1×10^6 rat thymus cells following a 16 hour preincubation with thymic or splenic extract 5, fraction III, and culture in "extract-free" medium.

DISCUSSION

From the results presented in this chapter it would seem that, at least in the rat, increased PHA responsiveness is not readily demonstrable following incubation of various unfractionated lymphoid cell populations with crude thymic extracts.

The most reproducible effect of the thymic and splenic extracts was that of decreasing the amount of tritiated thymidine incorporated by PHA-stimulated and unstimulated cells. Reduction in isotope incorporation occurred when the cells were cultured with tissue extract throughout the duration of the culture. The inhibitory effect of the extract appeared to be largely reversible as cells precultured with extract and resuspended in medium without extract showed little difference in isotope uptake compared to those cells cultured entirely without extract. The inhibitory effect of thymic extracts has been observed by others in mitogen stimulated (Touraine, J.L. et al 1974) (Rotter, V. and Trainin, N. 1975) and unstimulated cells (Trainin, N. et al 1975). Trainin and his associates found that splenic extracts neither inhibited nor enhanced tritiated thymidine incorporation in mitogen stimulated (Rotter, V. and Trainin, N. 1975) and unstimulated cells (Trainin, N. et al 1975). In the study described in chapter five, however, very little difference was found between the inhibitory effect of the thymic and splenic extracts.

Suggestive results for an effect of the thymic extract in increasing the responsiveness of cells to PHA was obtained from experiments (2) and (5). In experiment (2) a slight increase in the PHA response was found in both thymectomized rat spleen cells and bone marrow cells during incubation with 56 μg. thymic extract batch 3, fraction I. In experiment (5) thymectomized rat spleen cells were cultured with two concentrations of thymic extract batch 4, fractions I and II. A 9 hour preculture with 22.5 μg. per culture thymic extract

followed by culture in "extract-free" medium led to a PHA response greater than that in cells precultured without extract. Those cells precultured with 22.5 μg. F_{II} thymic extract showed a greater increase in PHA response than the cells precultured in the same amount of the F_I extract. Preculture with 90 μg. of extract F_I led to no change and 90 μg. F_{II} led to a slightly decreased PHA response. The result with 90 μg. extract could have been partly due to inhibition by extract carried over into the culture when the cells were resuspended. The cells were only centrifuged once and therefore not all of the preculture medium would have been removed and so the "extract-free" medium would, in fact, have contained small amounts of extract.

In experiments (8) and (9) increased isotope incorporation in both PHA- stimulated and unstimulated bone marrow and thymus cells were seen at certain concentrations of both the thymic and splenic F_{III} extract. The nature of this increase in isotope incorporation was not known. Positive effects of spleen extracts have been found by other workers (Cohen, G.H. et al 1975) especially those extracts purified to F_{III} (Scheid, M.P. et al 1973).

One result obtained from bone marrow cell cultures in which erythrocytes were not added was that isotope uptake in cultures without PHA was considerably increased in the presence of thymic extract (results not presented in this thesis). An effect of tissue extract in increasing isotope uptake in unstimulated cells was also seen in cultures containing erythrocytes, see experiments (3) and (9). This effect was thought to be most likely due to better survival of the cells in the presence of tissue extract but could also have been due to a lymphokine-like activity or a lymphocytopoietic activity. Cell counts before and after incubation with thymic and splenic extracts showed no

consistent changes in the number of viable cells (unpresented results).

There were several possible reasons why largely nagative results were found in the attempts to increase PHA responsiveness by the incubation of lymphoid cells with thymic extracts. The simplest reason is that incubation with thymic humoral products does not convert cells, incapable of responding to PHA, into cells capable of response to PHA. Assuming that such a transition is possible, however, perhaps the most likely reason was that the number of cells capable of responding to a thymic humoral influence, by becoming PHA responsive, in the bone marrow, spleen and thymus were too few to register in the PHA assay. Any small increase in PHA response might have been hidden by the pre-existing response to PHA. Cell fractionation studies were not carried out and this approach might have succeeded in finding a more sensitive cell population. It is also possible that the length of time following thymectomy might be important in regulating the number of cells sensitive to a thymic humoral factor. (See discussion chapter four). Indeed, thymectomized spleen cell cultures (experiments (2) and (5)), gave results showing small increases in PHA responsiveness after incubation with thymic extracts. The donors used in experiments (2) and (5) were the youngest of the donors used throughout the experiments described in this chapter.

The thymic extract might have been inactive. Three batches of extract were used in the experiments described in chapter five. Batch 4 was prepared solely from donor rats syngeneic to those used in the lymphoid cell cultures. Extract batch 3 was prepared from random-bred Sprague-Dawley rats (OLAC strain) and batch 5 from a mixture of OLAC and ADRI rats (table 5.1). Slight histocompatibility differences in

the thymic extract - donor rats, however, would be most unlikely to make the extract much less effective than a syngeneic extract as thymic extracts are believed to be neither strain nor species specific in their immunological effects (Trainin, N. 1974). Details of the tissue extracts used in each experiment are shown in table 5.14. Each extract was stored at - 20°C for various times before use. This storage could have reduced or destroyed the activity of the extract.

Table 5.14 Summary showing the length of time the various extracts were stored before use in lymphocyte culture.

Experiment	Tissue	Extract		
		Batch	Fraction	Age of extract when used (days)
1	thymus spleen	3	I	47
2	thymus	3	I	54
3	thymus	3	I	68
4	thymus	4	I II	4
5	thymus	4	I II	32
6	thymus spleen	5	III	93
7	thymus spleen	5	III	108
8	thymus spleen	5	III	118
9	thymus spleen	5	III	170

CHAPTER SIX

ORGAN CULTURE STUDIES

INTRODUCTION

Tissue culture and the thymus

Since the early part of this century and with the development of tissue culture techniques many attempts have been made at organ culture of the thymus (Pappenheimer, A.M. 1913) (Popoff, N.W. 1926) (Deanesly, R. 1929) (Emmart, E.W. 1936) (Murray, R.G. 1947) (Ball, W.D. and Auerbach, R. 1960) (Mandel, T. and Russell, P.J. 1971) (Van den Tweel, J. 1971) (Alm, G.V. and Sallström, J.F. 1972) (Robinson, J.H. and Owen, J.J.T. 1976). The above authors tried mainly to answer basic questions concerning the histology and histogenesis of the thymus. Pappenheimer was concerned primarily to investigate the possible secretory function of the thymus (Pappenheimer, A.M. 1913). His histological studies, however, provided no clear cut evidence of secretory function in any cell type within the organ. Popoff cultured pieces of thymus from newborn, half-grown and fully-grown rabbits in plain and carminized plasma containing bone marrow or embryonic extract (Popoff, N.W. 1926). He reported an early necrosis in the deeper part of the explant and a massive emigration of small cells (lymphocytes). This was followed by the development of an epithelial cell layer in the periphery and mitoses appearing throughout the epithelium. By 20 days the epithelium had undergone progressive atrophy and overgrowth by connective tissue had occurred. Popoff concluded that this study produced no evidence for the theory that small thymus cells (lymphocytes) were modified epithelial cells. Ball and Auerbach cultured non-lymphoid, embryonic thymuses in order to investigate whether the subsequent development of lymphocytes within the thymus was due to immigration from an extraneous source or due to the transformation of a native cell type (Ball, W.D. and Auerbach, R. 1960) (Auerbach, R. 1961). Their method, however, was

inadequate to answer that question as it failed to exclude the possibility of an early entry of stem cells into the thymus.

Several methods for organ culture of embryonic thymuses have been developed which allow lymphoid development in vitro. Ball and Auerbach cultured individual thymuses from day 12 mouse embryos in open filter-plexiglass wells (Ball, W.D. and Auerbach, R. 1960). A hole was drilled in the centre of a strip of plexiglass and a 20 mμ thick millipore filter cemented under the hole. This assembly was then placed into a tissue culture dish. Culture medium consisted of 40% Tyrode's, 40% horse serum and 20% chicken embryo juice plus antibiotics. Explants were incubated at 37°C in a water-saturated atmosphere of 95% air/5% CO_2 or 95% O_2/5% CO_2. Thymuses maintained in culture for 10 days or longer showed significant numbers of lymphocytes.

Mandel and co-workers (Mandel, T. and Russell, P.J. 1971) (Mandel, T. et al 1972) using the filter well assembly of Auerbach (Ball, W.D. and Auerbach, R. 1960) studied the development of lymphoid and epithelial cells in 12 - 13 day old foetal mouse thymuses under in vitro conditions. The culture medium consisted of Eagle's basal medium supplemented with 10% horse serum, 5% chick embryo extract and antibiotics. The organs were grown in a humidified atmosphere of 5% CO_2 in air with a change of medium every three days. The explanted thymuses consisted initially of undifferentiated epithelial cells and some large lymphoblasts. During the first week in culture the explants flattened on to the millipore filter and increased markedly in area although very little cellular outgrowth occurred. The epithelial cells differentiated rapidly but dividing epithelial cells were seen at all stages of the culture. During the second to the tenth day of culture the epithelial cells differentiated into small and medium lymphocytes, and lymphocytes became the most common cell type by the end of the first week. After

this period of rapid proliferation, lymphopoiesis stopped and the number of small lymphocytes gradually decreased, however even after 28 days culture viable small lymphocytes were seen.

In order to determine whether the cessation of lymphopoiesis in foetal thymus cultures was due to an exhaustion of the proliferative capacity of the stem cells or whether it was due to the failure of the epithelial microenvironment to sustain it, thymuses which had been maintained in vitro for fourteen days were grafted beneath the kidney capsule of two-week-old mice. Within 24 hours after grafting, most of the lymphocytes had disappeared from the graft which consisted mainly of epithelial cells. During the second and third day the graft consisted of large epithelial cells, many of which were in mitosis. During the third day large lymphoblast-like cells entered the graft and these cells began to divide and differentiate so that, by the end of the first week a typical, although small, thymus was present. Long term (6 - 10 week) grafts had the morphology of normal post-natal mouse thymus grafts. When "cultured" grafts were compared to normal grafts the only apparent difference was the poor development of the medulla and the paucity and poor differentiation of medullary epithelial cells in "cultured" grafts.

A similar study was undertaken by Alm and Sallström who cultured embryonic chicken thymus (Alm, G.V. and Sallström, J.F. 1972) (Sallström, J.F. and Alm, G.V. 1973). Their experiments were designed to assess the role of the thymic microenvironment in the morphological and functional differentiation of lymphocytes from the first lymphoid precursors. The organ cultures were a modified Trowell type (Jensen, F.C. et al 1964). 60 mesh, stainless steel grids were placed in Falcon plastic, 35 x 10 mm. tissue culture dishes, each containing 2 ml of culture medium. A millipore filter, pore size 0.45μ was placed on top of the grid at the gas-medium interface. The filters were covered with slices of spongostan

gelatin foam which were allowed to become saturated. One whole thymic anlage was then placed on each grid. The cultures were incubated in gas-tight chambers at 37.5°C in a water-saturated atmosphere of 5% CO_2, 57% O_2, 38% N_2. Half of the medium was replaced with fresh medium at day 5, if the culture time exceeded six days. Several different media and supplements were tested and Waymouth's MB 752/1 plus 10% chicken serum was found to give the best results. The thymic anlagen of 10-day-old chick embryos were small and contained relatively few dells resembling lymphoblasts and no lymphocytes. The first few small lymphocytes were noted after 2 to 4 days in culture. The proportion of small lymphocytes increased rapidly and these cells were the most common cell type after culture for 10 days. These lymphocytes were found to be reactive to the T cell mitogens PHA and ConA (Sallström, J.F. and Alm, G.V. 1973). Robinson and Owen have recently reported a similar study in the mouse (Robinson, J.H. and Owen, J.J.T. 1976).

Maintainance of the thymus in organ culture has been used by several workers in investigations of thymic function.

During an investigation into the "Lymphocytosis Stimulating Factor" (LSF), extractable from human and mouse thymus and present in the plasma in certain disease states, Metcalf carried out some organ culture studies (Metcalf, D. 1956 c). Metcalf used Maitland-type tissue culture techniques. Mouse thymuses were aseptically excised, washed in Earle's solution and coarsely minced with sharp scissors. The pieces were transferred to 5 x $\frac{5}{8}$ inch roller tissue culture tubes. Each tube contained either three 8-day-old or ten 2-day-old thymuses. Two ml. of Earle's solution containing 50 units each of penicillin and streptomycin was then added to each tube. The tubes were incubated at 36°C and rotated at 60 rotations per hour. After one to five days in

culture the total LSF present in the supernatant fluid and organ-cultured thymus was found to be greater than in control thymuses not organ cultured. This was thought to indicate production and release of LSF into the medium.

Desomer and co-workers suspended small pieces of calf thymus in Hank's buffered solution and gently shook the cultures at 37°C for 48 hours (Desomer, P. et al 1963). The harvested fluid was centrifuged twice at 3,000 rpm for 30 minutes before use. Spleen cultures were used as controls in the ensuing tests. Thymus culture fluid was injected intraperitoneally into five-week-old normal mice and found to elevate the total number of white blood cells compared to mice injected with spleen culture medium. This increased total WBC level was due mainly to an increase in absolute lymphocyte numbers. When neonatelly thymectomized mice were injected with thymus culture medium from three weeks of age, lymphocyte counts returned to normal and treated mice showed normal body weights and did not succumb to wasting. These mice were also resistant to the normally fatal consequences of infection with type 4 adenovirus. It was later reported that Desomer was unable to repeat these observations (Trainin, N. 1974).

Globerson and Auerbach used organ culture methods to study the reactivation of immunocompetence of irradiated mouse spleen cells (Globerson, A. and Auerbach, R. 1967). The competence of spleen cells to invoke an in vitro G v H reaction was lost upon sublethal irradiation. When such spleen explants were grown in the presence of thymus, but not in the presence of a variety of other tissues, restoration of competence occurred. A humoral role for the thymus was suggested by experiments showing that this thymic activity could cross a millipore membrane of 0.3 or 0.1μ porosity. When lethal doses of irradiation were used,

reactivation of immune competence did not occur unless both thymus and bone marrow were present in which case the competent cells appeared to come from the bone marrow.

A similar study investigated the role of the thymus in the development of immunocompetence of embryonic liver cells in vitro (Umiel, T. et al 1968). Embryonic liver cells did not have the capacity to induce splenomegaly when taken from embryos directly, nor did they acquire this capacity when cultivated as organ cultures, by themselves or in the presence of adult spleen or lung, for four to six days. The capacity to induce splenomegaly was acquired, however, when liver explants were cultivated for several days in combination with thymus tissue. The strain combinations used ruled out the possibility that thymus cells were active in producing the G v H reaction, thus showing that competent cells were of liver origin. The thymus and liver were not separated by millipore membranes and therefore a purely humoral mechanism cannot be claimed for the increased immune reactivity of the liver cells.

Reese and Israel performed some simple tissue culture experiments to investigate the role of a possible thymic humoral factor in the restoration of the ability of neonatally thymectomized mice to respond to sheep erythrocytes.-(Reese, A.J.M. and Israel, M.S. 1967) (Reese, A.J.M. and Isreel, M.S. 1969). Three whole neonatal mouse thymuses were explanted onto the sides of 10 x 1.3 cm. test tubes which were then incubated for one hour at 37°C to ensure attachment to the glass surface. One ml. of medium 199 containing 20% horse serum, penicillin, streptomycin and nystatin was then added and the tubes were incubated at 37°C at 5° to the horizontal. The medium was decanted at periods between 3 and 7 days after explantation and stored at 4°C. The medium was injected i.p. into neonatally thymectomized mice aged between 5 - 30 days in 0.3 ml. and later 0.6 ml amounts on five occasions within ten days. Similar neonatally thymectomized mice were given identical doses of unused growth

medium and used as controls. Between 56 and 70 days later the antibody forming response to sheep erythrocytes was measured by the Jerne plaque technique. 51 thymectomized mice given i.p. injections of thymus organ culture fluid showed no increase in plaque counts compared to 45 control mice given unused growth medium. The plaque counts of treated mice were similar to those of untreated thymectomized mice. The negative results obtained with thymus organ medium injected mice could have been due to the small total amounts of medium (thymus factor) injected.

In order to check the viability of thymuses grown in organ culture neonatally thymectomized mice were grafted at the age of 28 - 42 days with explants of whole syngeneic thymus grown in culture. Although there appeared to be good preservation of architecture in sections cut from explants grown in culture for up to 28 days no thymus grown for more than 9 days survived transplantation. Twenty two neonatally thymectomized mice received whole syngeneic thymus grafts from thymuses grown in culture for 1 - 9 days. All these mice showed considerable restoration of immune competence as judged by increased plaque counts in the Jerne assay thus confirming the viability of the organ cultured thymuses.

In a study where thymus and lymph node fragments were co-cultured it was found that normal thymus fragments from 1- to 2-week-old rabbits enhanced de novo synthesis of 7S Immunoglogulin by immune popliteal lymph node fragments (Wolf, B. 1968). A more recent report from Wolf (Wolf, B. 1975) described an in vitro system in which lymph node cells are sensitized with diptheria toxoid in vitro to produce a secondary response. In this system potentiation of antibody synthesis occurred when lymph nodes were co-cultured with normal thymus, thymus that had been heated at 50°C for 30 minutes and thymus separated by a

millipore diffusion chamber. Bovine thymosin, fraction 3 or 4 (Goldstein, A.L. et al 1972) was also found to enhance the secondary response in vitro either when included in the culture medium or when added to the antigen and washed out of the culture, after two hours. Controls for this study included co-incubation with normal spleen fragments, in which case antibody synthesis was depressed. Controls for calf thymosin were identically derived extracts prepared from calf spleen and brain. The brain fraction showed no activity and the spleen fraction only a small activity.

Mandel and co-workers assayed the medium from their thymus organ cultures for the presence of a competence-inducing factor (Mandel, T. et al 1972). They collected medium from the cultures at three day intervals and batches of the same age were pooled, dialysed against PBS and lyophilized. The dried material was then redissolved in EMEM to a concentration five times that of the original. For controls, medium was obtained from cultures of foetal mouse liver, lung and kidney and processed identically. The concentrated media were sterilized by filtration and used as a 2½% supplement to the tissue culture medium in the assay. To test for restoration of immune competence, the in vitro response of mouse spleen cells to sheep erythrocytes was used as described by Marbrook (Marbrook, J. 1967). In this assay 20×10^6 spleen cells were immunized in vitro with 3×10^7 sheep erythrocytes and after four days the number of antibody-forming cells was assessed by a modified Jerne plaque assay. When spleen cells from neonatally thymectomized mice were exposed for the entire culture period to a 2½% supplement of organ culture medium, restored ability to respond to sheep erythrocytes was noticed only with the thymus medium supplement. Other supplemented media were completely ineffective except for foetal liver where a slight restorative effect was noticed. The restorative effect of thymus supplements was only found

in medium taken from 3, 6 and 9 day organ cultures, medium taken from later cultures was ineffective. Care was taken to exclude the possibility that the FCS used in the assay was responsible for the observed restoration.

A method for preparing monolayer cultures of rat or mouse reticulum cells was developed by Wekerle and co-workers (Wekerle, H. et al 1973). These workers had previously found that thymus lymphocytes can become autosensitized to reticulum cells and then interfere with the growth of the reticulum cells (Cohen, I.R. and Wekerle, H. 1973). It was therefore found necessary to deplete the culture of lymphocytes. Fresh thymus glands in PBS at $4^{o}C$ were minced to a fine pulp, washed with PBS and treated with 0.3% trypsin for 10 minutes at room temperature under gentle agitation. The cells in suspension were mostly lymphocytes and were discarded. The reticulum cells were then brought into suspension by more vigorous trypsinisation conditions. The remaining lymphocytes were eliminated by culturing the cells in 100 mm plastic petri dishes at a concentration of 30×10^{6} cells per ml. in 10 ml. Waymouth's medium. This medium does not support the growth of lymphocytes. Waymouth's medium was exchanged for Eagle's medium after 24 hours for mouse or 48 hours for rat cultures and subsequently the Eagle's medium was replaced with fresh Eagle's medium every third or fourth day. The reticulum cells adhered to the petri dishes within the first few hours of culture and proliferated to form a confluent monolayer by one week. The following cell types were described in early cultures: (1) Large cells with irregular shapes, oval nuclei containing two dense nucleoli. This was the most common cell type. These cells contained a large number of P.A.S.-staining vacuoles, especially in the perinuclear region. (2) Smaller dendritic cells containing P.A.S.-positive material which was not restricted to vacuoles but distributed in the cytoplasm (3) Epithelial

cells which were P.A.S.-negative. From the second week of culture clones of spindle-shaped myoblasts appeared, these fused to contracting muscle fibres after the tenth day of culture.

The above culture system had been devised in order to investigate the role of thymus reticulum cells in the differentiation of thymic-dependent lymphocytes. Two tests of immunocompetence were used: the ability to respond to ConA and the ability to become sensitized in vitro against foreign antigens, as shown by the enlargement of popliteal lymph nodes when these lymphocytes are injected into the footpads of syngeneic animals. In the first test thymus-deprived or normal mouse spleen cells were incubated for 24 hours with reticulum cell or fibroblast cultures obtained from Lewis rats. The lymphocytes were then removed from the monolayer and cultured with and without ConA. It was found that spleen cells from thymus-deprived mice cultured with rat fibroblast cultures did not respond to ConA (stimulation index, SI = 0.81). Intact mouse spleen cells cultured on fibroblasts had a SI of 1.63 and spleen cells from thymus-deprived mice cultured on rat thymus reticulum cultures had a SI of 1.59. In the second test, spleen cells from control or thymus-deprived mice were sensitized against monolayers of rat fibroblast or thymus reticulum cells for 24 hours in vitro. The lymph node assay in syngeneic mice was used to measure the degree of sensitization against the rat cells. It was found that spleen cells from normal mice produced the same lymph node index, about 6.5, whether they were sensitized against rat fibroblasts or thymus reticulum cells. In contrast, spleen cells from thymus-deprived mice, sensitized against fibroblasts, showed a markedly decreased ability to stimulate a lymph node response (index = 2.7). Incubation with thymus reticulum cells, however, restored the response completely (index = 6.7). Both the above tests produced the same

results when immunocompetent mouse spleen cells were cultured with mouse thymus reticulum cell cultures. The differentiating effect of the thymus reticulum cells was attributed to two possible mechanisms, either cell to cell contact between the reticulum and spleen cells or as a result of a factor secreted by the reticulum cells acting on the spleen cells, or both these mechanisms.

A humoral factor originating from thymus epithelial cell monolayer cultures and active in increasing the ability of bone marrow cells to form rosettes with sheep erythrocytes was found by Pyke and Gelfand (Pyke, K.W. and Gelfand, E.W. 1974). They established epithelial outgrowths from explants of human thymus obtained from children aged one week to thirteen years undergoing heart surgery and maintained these cultures for up to three months. Thymus fragments were minced, washed several times and cultured in plastic flasks with Dulbecco's modification of Eagle's medium containing antibiotics and 30% foetal bovine serum (FBS). After 5 - 7 days culture an epithelial outgrowth was observed and the medium was then replaced twice weekly until a confluent monolayer was observed and then the concentration of FBS was reduced to 20%. Fibroblasts were present in all cultures and persisted, in most cases, as a 5 - 10% contamination of the monolayer. When medium was collected from the cultures it was centrifuged at 550 g. for 10 minutes at 10°C and the supernatant was stored at -20°C.

Bone marrow cells from human donors were run on Isopaque-Ficoll gradients to remove erythrocytes and enrich for lymphocytes. The rosette-forming ability of the marrow cells was enumerated after incubation for three hours at 37°C with fresh medium, medium from thymus cultures and medium from Chang human liver cells. The results suggested that only conditioned medium from the epithelial monolayers was capable of increasing the capacity of unfractionated human marrow

cells to form rosettes with sheep erythrocytes. It was found that the most effective thymus conditioned medium was that obtained when thymic lymphocytes were no longer present in the cultures. Non-lymphoid cells (Chang human liver), peripheral blood lymphocytes, tonsillar lymphocytes and cells from an established B cell line, were unaffected by the conditioned medium.

Further evidence of the functional activity of human epithelial cells in culture was provided by Papiernik and co-workers (Papiernik, M. et al 1975). Cultures were established from biopsy samples from children aged nine months to nine years who were undergoing open heart surgery. About 30 thymus fragments were explanted into Falcon plastic flasks and cultured in Eagle's medium containing 10% human AB serum. Human serum was obtained from individuals aged over 60 years to avoid the high thymic factor activity found in younger subjects (Bach, J.F. and Dardenne, M. 1972). Cultures were kept at 37°C and the medium was renewed twice each week. Rings of epithelial cells were visible at the explant periphery within two to three days; after seven to eight days no more lymphocytes were found in culture. Control cultures of human spleen and fibroblasts were also carried out. Thymic activity was detected by the rosette inhibition test (Bach, J-F. et al 1971 b) using anti-θ serum and spleen cells from CBA adult mice thymectomized 10 - 20 days before sacrifice. Direct incubation of these spleen cells with cultures of thymic epithelium but not others increased the sensitivity of the test spleen cells to anti-θ serum. An incubation time of only 15 minutes was required to effect this change. The duration of the epithelial cell culture was found to be critical, 46% of the tests were positive using 7 - 15 day cultures whereas no positive results were obtained after 15 days despite no electron microscopic evidence of abnormalities. In order

to investigate whether the thymic activity resulted from cell to cell contact or was humoral in nature spleen cells were enclosed within a millipore diffusion chamber of pore size 0.45μ and cultured along with the epithelial cells. Since these cells similarly gained increased sensitivity to anti-θ serum it was concluded that the epithelial cells modified the spleen cells by the production of a humoral factor. The authors did not say whether they harvested supernatants from epithelial cell cultures and incubated spleen cells in harvested media which would have been a simpler way of demonstrating a humoral factor.

EXPERIMENTAL AIMS.

The object of the work described in this chapter was to develop a method for obtaining thymic humoral factor(s) and then to evaluate such a factor (s) by the use of a PHA assay. Organ culture is a technique that aims to preserve the functional relationship of the different cell types in vitro. As such, it was hoped that viable thymus explants would synthesize and release any humoral products into the surrounding medium.

A simple method of organ culture was used - a modified Trowell type. The viability of the explants was assessed by histological and transplantation studies. Attempts were made to assay the medium for thymic humoral factor activity by using thymectomized rat spleen cells, bone marrow cells and thymus cells. Both "continuous culture" and "preculture" techniques were used to investigate whether medium from thymus organ cultures had an activity that increased the PHA response of lymphoid cells.

METHODS

I. Organ culture technique

Stainless steel expanded metal grids (1.5 mm. mesh) (Expanded Metal Company), with a surface area of approximately 400 mm^2 were made by bending over the two ends of a flat piece of metal to make legs about 6 mm high. Sterile grids were placed into plastic petri dishes (35 x 10 mm) (Falcon Plastics) and a piece of filter paper was placed on top of each grid to act as a support for the explants. The medium was then added to such a level that the filter paper became saturated. This occurred when between 5 and 6 ml. of medium had been added. Both medium 199 and EMEM were used in the organ cultures. Medium 199 was used in the in vivo study described in Reconstitution Experiment Two, chapter four. EMEM was used in the in vitro lymphocyte cultures described in the present chapter. Various concentrations of FCS and ADRI rat serum were used to supplement the medium and these are detailed in the text.

The thymuses and spleens used for organ culture were obtained from male rats aged 40 to 70 days. The donor rats were killed by ether inhalation and the thymus and spleen carefully dissected out using aseptic technique. The organs were washed with saline and transferred to a sterile glass petri dish containing medium. The connective tissue and the fat surrounding the organ were then removed and the tissue cut into several thin slices using a razor blade. The slices were cut into approximately 1 mm. cubes and six explants placed onto the filter paper support. The petri dishes were placed into a plastic box which contained two or three open dishes containing sterile distilled water. The box was sealed and gassed with 5% CO_2 in air and placed into an incubator at 37°C. Each day the box was regassed. After three days the medium was harvested and replaced with fresh medium. The harvested medium was transferred to sterile 10 ml. glass centrifuge tubes and centrifuged at room temperature

for 15 minutes at 3,000 rpm; the supernatant was placed into sterile universal containers. In experiment (1) the medium was stored at -20°C for ten days before use, in all the other experiments described in this chapter the medium was stored at 4°C and used within 24 hours.

II. Lymphocyte culture

The lymphocyte culture methods used in this chapter were essentially those described in chapter two. Tritiated thymidine of high specific activity (22.3 Ci/m.mol.) was used to assess the response to PHA in experiment (1). In all the other experiments described in this chapter the low specific activity tritiated thymidine (5 Ci/m.mol.) was used.

EXPERIMENTAL DESIGN AND RESULTS

I. Histological study of organ cultured explants.

Organ cultured explants of thymus and spleen were examined by both light and electron microscopy.

Photomicrographs of standard haematoxylin and eosin stained sections of thymus and spleen explants after three and six days in culture are shown in figures 6.1 to 6.4. The organ medium in this instance was medium 199 supplemented with 10% FCS. Considerable cellular disintegration was often seen in both the thymus and spleen explants, particularly in the centre of the explant. The examples reproduced here were amongst the better preserved specimens.

Examination of the explants by electron microscopy was undertaken primarily to study the viability of the different cell types within the thymus. The processing of the samples for electron microscopy and the sectioning and staining of the specimens was carried out by Mr. W. Hawkins of the Department of Clinical Surgery. The specimens were fixed in 2% gluteraldehyde in sodium cacodylate buffer, pH 7.4 for 4 to 5 hours and left overnight in cacodylate buffer. The specimens were postfixed in

osmium tetroxide, washed in distilled water, 5% acetone and 100% acetone, impregnated with araldite and embedded in fresh araldite. Thick sections and ultra thin sections (400 Å) were cut on an ultramicrotome and stained with uranyl acetate and lead citrate.

The electron micrographs showed that considerable cellular disruption had occurred in all the organ cultured thymuses. A typical area of necrotic cells is shown in figure 6.5. The centre of the explants was particularly necrotic, however, apparently viable lymphocytes, epithelial and reticular cells were present especially in the periphery, see figures 6.6, 6.7 and 6.8. Throughout the explant, epithelial and reticular cells appeared to be better preserved than lymphocytes, the latter being seldom found in a viable state. This is illustrated in figure 6.8. Further evidence for the viability of epithelial cells was the finding of desmosomes between adjoining epithelial cells. Desmosomes were seen in the thymus explants throughout the duration of the culture and even in explants cultured for ten days.

For the purposes of an investigation of possible thymus humoral factors, the functional integrity of the cells responsible for the synthesis and release of these factors was of paramount importance. These cells are thought to be epithelial cells for reasons that are discussed in chapter one and elsewhere. The apparent viability of epithelial-reticular cells during the period of organ culture as indicated by the electron microscopy studies was therefore consistent with the hope that thymic humoral products would be secreted into the medium during organ culture.

Figure 6.1

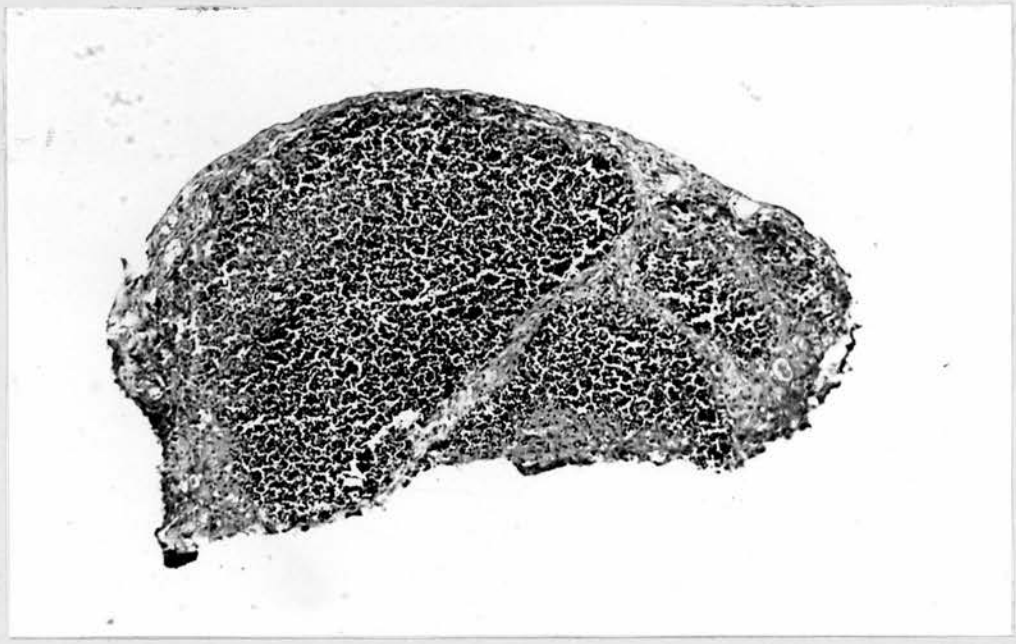
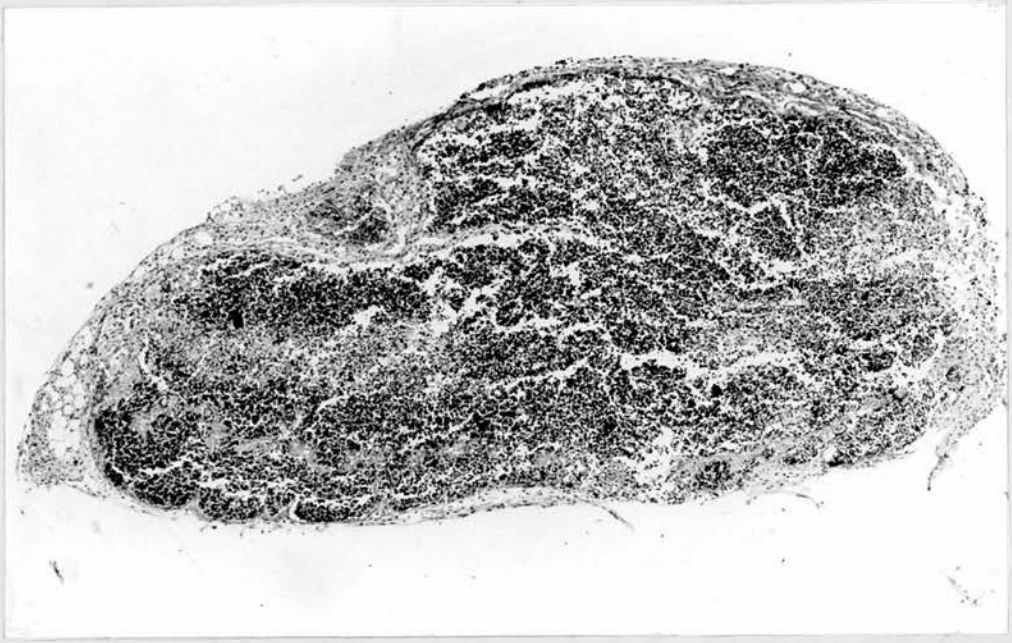


Figure 6.2



Thymus organ culture. Figure 6.1 explant after three days in culture (x 60)
Figure 6.2 explant after six days in culture (x 50).

Figure 6.3

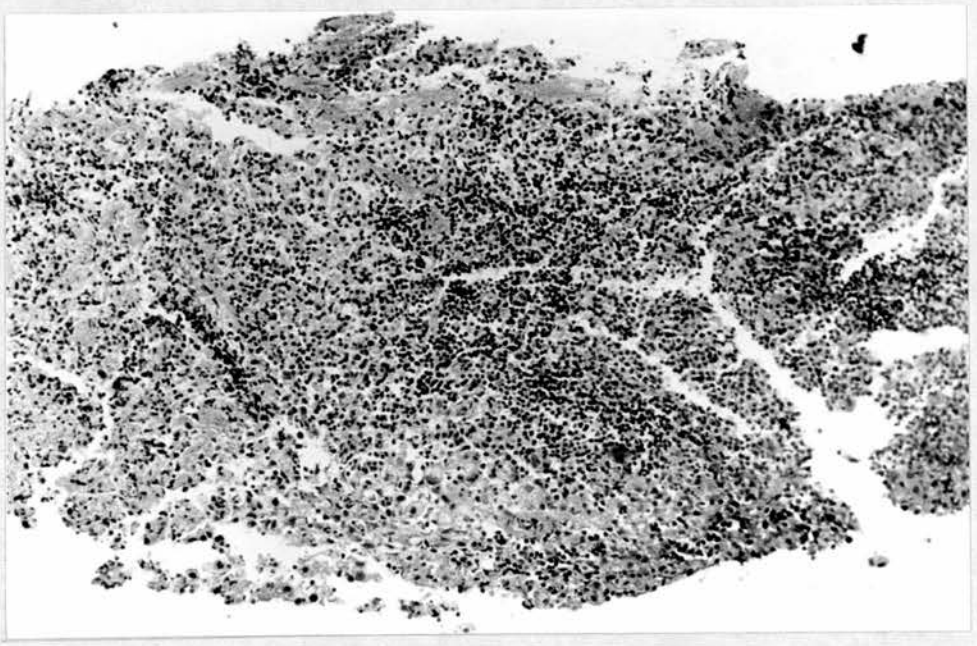
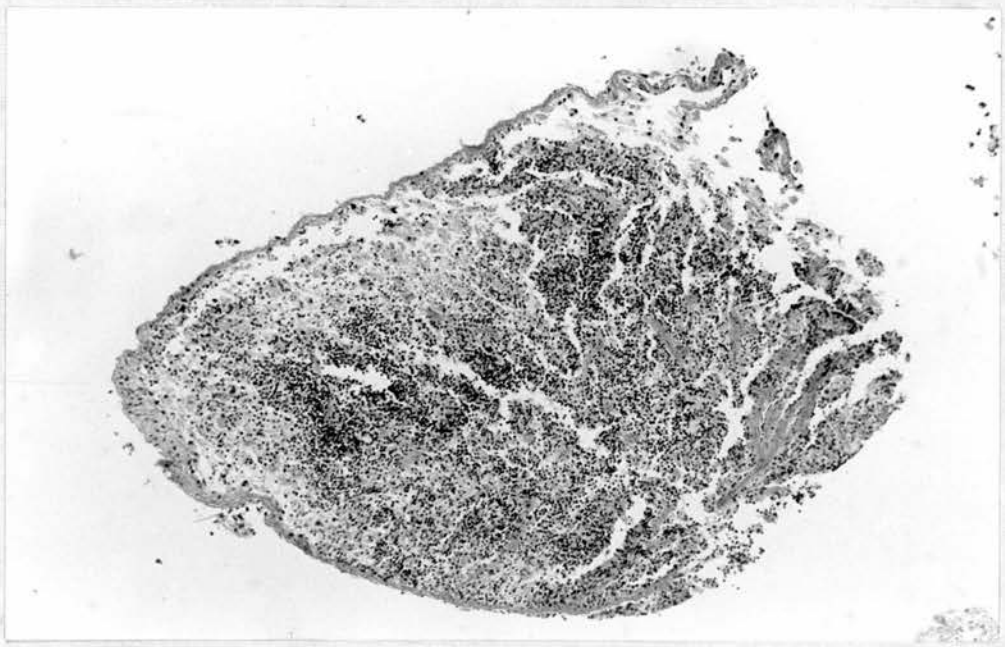


Figure 6.4



Spleen organ culture. Figure 6.3 explant after three days in culture (x 160). Figure 6.4 explant after six days in culture (x 60).

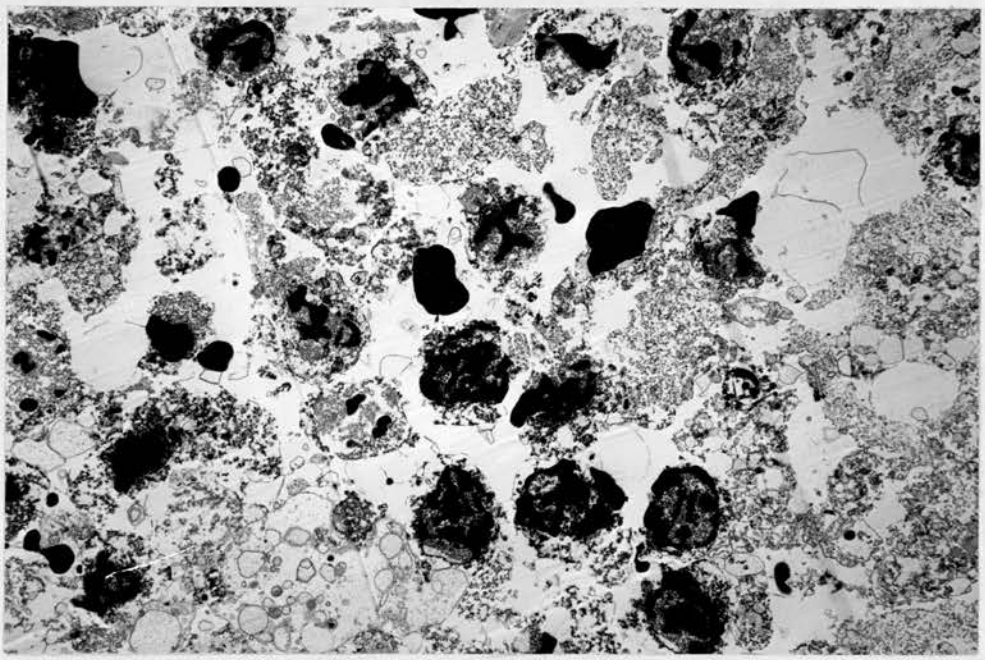


Figure 6.5 Thymus explant after organ culture for four days (x 4000). An area of necrotic cells.

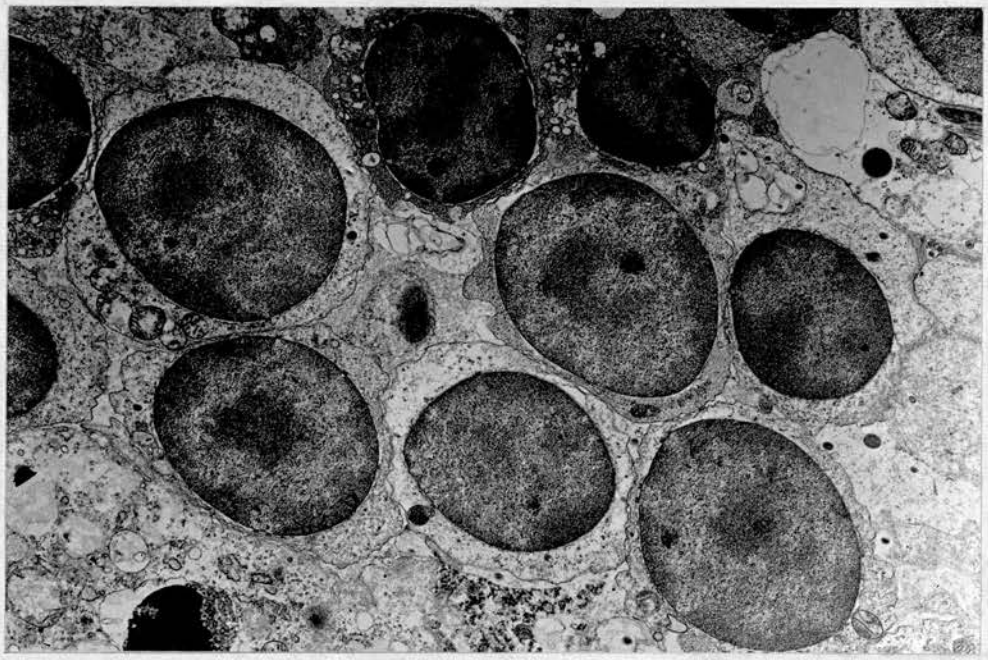


Figure 6.6 Thymus explant after organ culture for two days (x 10,000). An area showing viable lymphocytes with intact mitochondria.

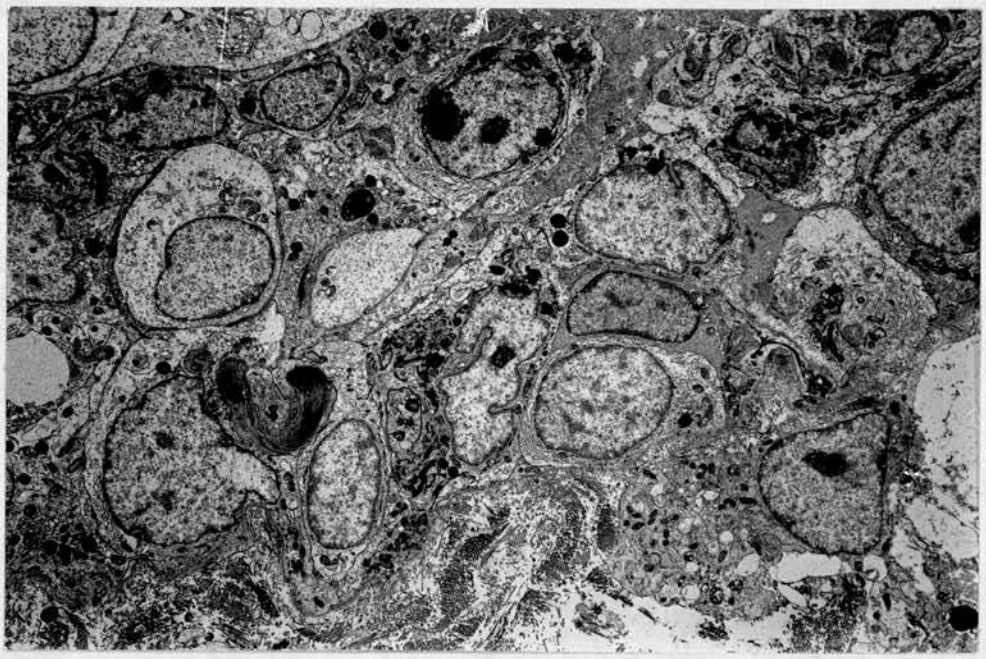


Figure 6.7 Thymus explant after organ culture for four days (x 4000). An area near the capsule showing lymphocytes and epithelial-reticular cells.

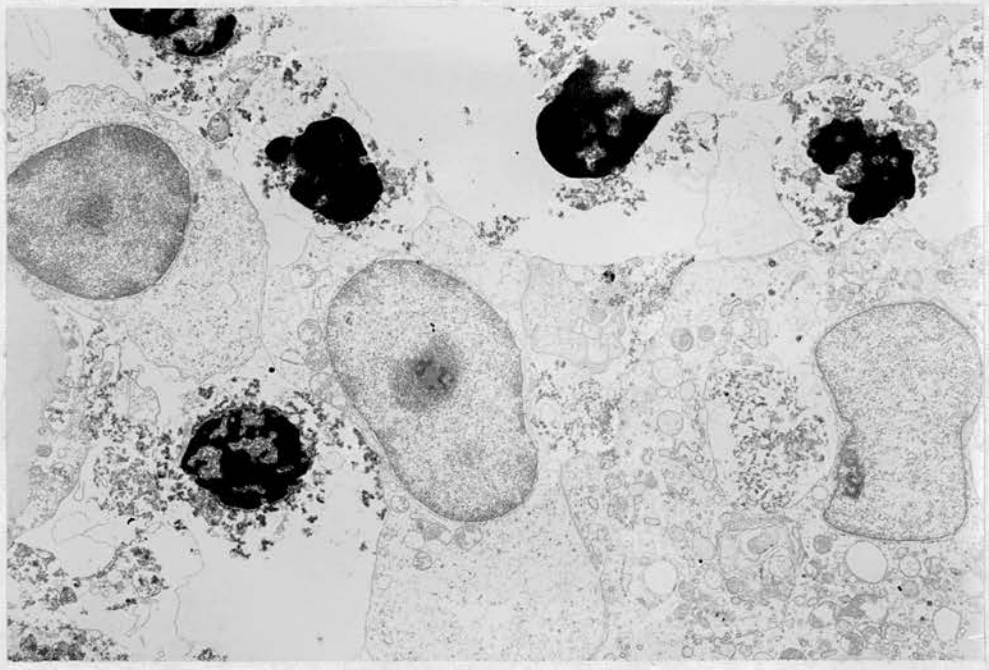


Figure 6.8 Thymus explant after organ culture for four days (x 6000). An area showing apparently viable cells and the pyknotic nuclei of dead cells.

II. Transplantation of organ cultured thymus explants.

The viability of thymus explants after varying periods in organ culture has been investigated by grafting the explant into a syngeneic host and, after a few weeks, removing the graft to see whether a histologically recognisable thymus graft was present (Reese, A.J.M. and Israel, M.S. 1967) (Mandel, T. and Russell, P.J. 1971).

Organ culture 16 (OC16) was designed to evaluate the viability of thymus explants after three and six days in organ culture. At the same time the medium harvested at day 3 and day 6 was assayed for the presence of a thymus factor as described in lymphocyte culture experiments (2) and (3). The medium used in OC16 was EMEM supplemented with 10% FCS and the thymus was obtained from a rat aged 52 days. After three days of culture the medium was harvested and pieces of organ cultured thymus were placed under the kidney capsule of two syngeneic male rats aged 55 days. Similarly, when the explants had been in culture for six days, with a change of medium at day 3, pieces of explanted thymus were grafted beneath the kidney capsule of three 43-day-old syngeneic male rats. Twenty two days after transplantation the thymus grafted rats were killed and examined for the presence of thymus grafts.

In one of the two rats grafted with thymus explants which had been cultured for three days a small but recognisable thymus graft was found. This is shown in figure 6.9. No graft was recovered from the other rat. When the rats grafted with thymus explants cultured for six days were examined only one rat was found to contain a lymphoid cell-containing graft. This is reproduced in figure 6.10.

This experiment, therefore, provided evidence that, at least after three days in culture, the thymus explant, in some cases, contained enough viable epithelial cells to enable the explant to reform after transplantation.

Figure 6.9

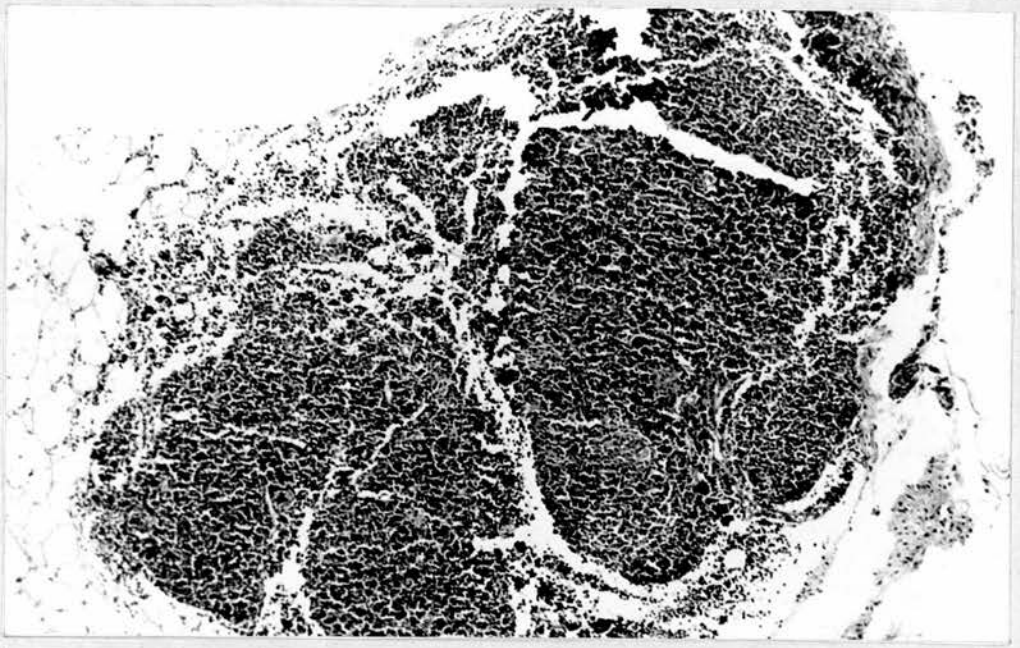
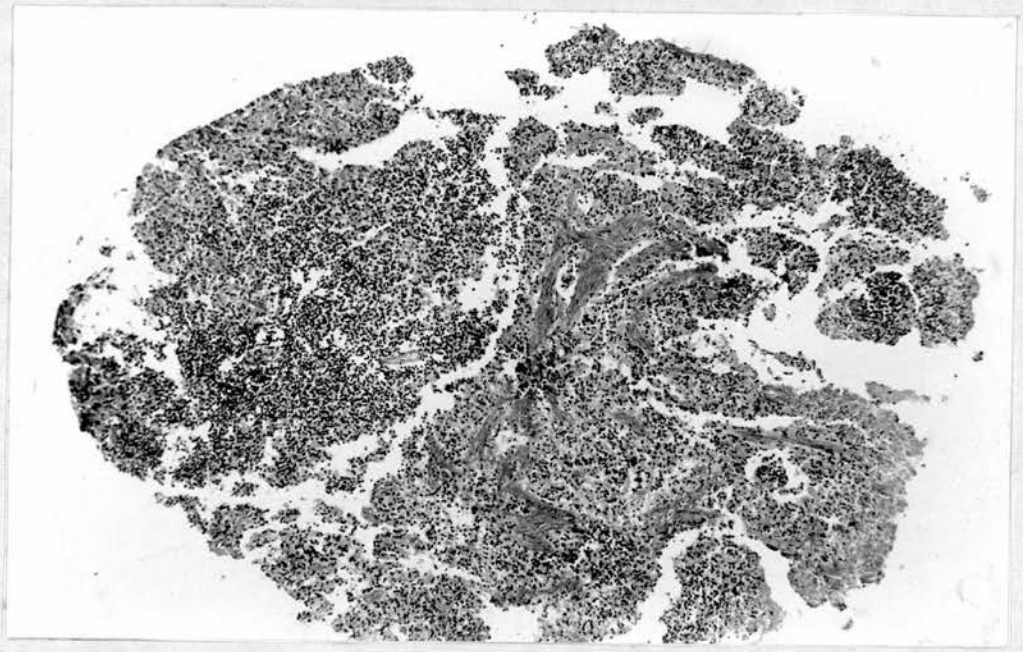


Figure 6.10



Thymus grafts recovered from rats 22 days following transplantation. Figure 6.9 explant grafted after three days in organ culture (x 60) Figure 6.10 explant grafted after six days in organ culture (x 60).

III. Lymphocyte culture studies.

In this section the effect of incubating lymphoid cells with medium obtained from thymus and spleen organ cultures was studied. The experimental design was similar to that of those experiments described in chapters three and five in which cells were incubated both in the continuous presence of thymus factor and also for various times before the removal of the thymus factor and the addition of PHA. As in the chapter on the in vitro effects of tissue extracts (chapter five), the spleen was used to provide a control for the effect of thymus organ cultured medium. Spleen organ cultures were performed at the same time as the thymus organ cultures and the medium was treated exactly as described for the thymus medium (see methods section).

Experiments

(1) The effect of an 8 hour preincubation and continuous culture with day 3 medium from thymus and spleen OC5, on the incorporation of tritiated thymidine in PHA-stimulated and unstimulated thymectomized rat spleen and bone marrow cells.

(2) The effect of (A) a 17 hour preincubation with day 3 medium from thymus and spleen OC16, followed by culture in fresh medium, and (B) a 17 hour preincubation and continuous culture with day 3 medium from thymus and spleen OC16, on tritiated thymidine incorporation in PHA-stimulated and unstimulated thymectomized rat spleen cells.

(3) The effect of (A) a 17 hour preincubation with day 6 medium from thymus and spleen OC16, followed by culture in fresh medium, and (B) a 17 hour preincubation and continuous culture with day 6 medium from thymus and spleen OC16, on tritiated thymidine incorporation in PHA-stimulated and unstimulated thymectomized

rat spleen cells.

(4) The effect of (A) a 17 hour preincubation with day 3 medium from thymus and spleen OC17, followed by culture in fresh medium, and (B) a 17 hour preincubation and continuous culture with day 3 medium from thymus and spleen OC17, on tritiated thymidine incorporation in PHA-stimulated and unstimulated rat thymus cells.

(5) The effect of (A) a 17 hour preincubation with day 3 medium from thymus and spleen OC18, followed by culture in fresh medium, and (B) a 17 hour preincubation and continuous culture with day 3 medium from thymus and spleen OC18 on tritiated thymidine incorporation in PHA-stimulated and unstimulated intact rat bone marrow cells.

Experiment (1) <u>The effect of an 8 hour preincubation and continuous culture with day 3 medium from thymus and spleen OC5 on the incorporation of tritiated thymidine in PHA-stimulated and unstimulated thymectomized rat spleen and bone marrow cells.</u>

Medium 199 containing 10 mM HEPES and 9% ADRI rat serum was used in organ culture OC5. After a three day culture, medium from both the thymus and spleen organ cultures was harvested, centrifuged and the supernatant stored at -20°C for ten days prior to use.

A series of culture vials were set up containing either 2.4×10^6 spleen cells or 3.7×10^6 bone marrow cells obtained from an 85 day old neonatally thymectomized rat. These cells were incubated in 1 ml. of medium 199 containing 10% FCS, supplemented with 0, 10, 40, 70% thymus or spleen OC5 medium. This design meant that although each 1 ml. culture contained 100 µlFCS, supplementation by different amounts of OC5 medium led to different concentrations of ADRI serum. Thus 10, 40 and 70% OC5 supplemented media contained respectively 0.8, 3.3 and 5.8% ADRI serum. After an 8 hour culture at 37°C, 4 µl. of PHA in 100 µl. of saline or 100 µl. saline were added and 100 µl. of an erythrocyte preparation were added to the bone marrow cells. Twenty three hours after the initiation of the culture erythrocytes were added to the vials containing spleen cells. The response to PHA was assessed by a 23 hour pulse of tritiated thymidine and the culture was terminated 64 hours after the addition of PHA.

The results of this culture are shown in table 6.1. Both the spleen and bone marrow cells were found to be practically unresponsive to PHA. The isotope uptake was found to decrease as the proportion of

medium supplement increased. This was not unexpected as the concentration of serum increased with increasing supplementation with organ culture medium and from the results obtained in chapter three it was known that when the serum concentration increased isotope uptake decreased. The effect of thymus and spleen organ culture medium can therefore only be compared "horizontally" and not "vertically" (see table 6.1). There was no significant difference between the isotope uptake in cultures supplemented with either thymus or spleen OC5 medium. Slightly greater isotope uptake occurred however, in both PHA-stimulated and unstimulated cell cultures containing spleen OC5 medium.

Table 6.1 Isotope incorporation in cultures of 2.4×10^6 thymectomized rat spleen cells and 3.7×10^6 thymectomized rat bone marrow cells following an 8 hour preincubation and continuous culture with day 3 thymus or spleen OC5 medium.

Cells (number).	OC5 day 3 medium supplement. (%)	Thymus medium			Spleen medium		
		4.0 µl PHA	0 µl PHA	SI	4.0 µl PHA	0 µl PHA	SI
		cpm., mean ± SD			cpm., mean ± SD		
TX rat spleen cells (2.4×10^6)	0	4,920 ± 359	5,789 ± 835	0.8	4,920 ± 359	5,789 ± 835	0.8
	10	2,797 ± 189	2,253 ± 198	1.2	3,300 ± 263	2,881 ± 121	1.1
	40	789 ± 101	512 ± 16	1.5	1,645 ± 40	903 ± 78	1.8
	70	316 ± 24	225 ± 10	1.4	1,126 ± 42	381 ± 69	3.0
Tx rat bone marrow cells (3.7×10^6)	0	8,557 ± 871	10,351 ± 2,351	0.8	8,557 ± 871	10,162 ± 2,351	0.8
	10	7,239 ± 740	9,671 ± 493	0.7	9,104 ± 1,426	6,711 ± 1,328	1.4
	40	2,966 ± 200	2,310 ± 1,660	1.3	5,387 ± 680	2,372 ± 572	2.3
	70	1,126 ± 323	1,668 ± 424	0.7	1,295 ± 258	1,412 ± 918	0.9

Experiment (2) <u>The effect of (A) a 17 hour preincubation with day 3 medium from thymus and spleen OC16, followed by culture in fresh medium and, (B) a 17 hour preincubation and continuous culture with day 3 medium from thymus and spleen OC16, on tritiated thymidine incorporation in PHA-stimulated and unstimulated thymectomized rat spleen cells.</u>

This experiment was the first half of an investigation which studied the effect of medium obtained from thymus and spleen OC16 on thymectomized rat spleen cells. Experiment (2) studied the medium harvested at day 3 whilst experiment (3) studied the medium harvested at day 6. The organ culture medium was EMEM containing 10% FCS. Medium was harvested after three days in culture and was used one day later in the lymphocyte culture. When this medium was taken from the organ culture it was replaced with fresh medium and after a further three days in culture the medium was again harvested and used, one day later in lymphocyte culture experiment (3).

18.2×10^6 spleen cells from a 48 day old neonatally thymectomized rat were cultured in plastic universal containers (UC's) containing 6.6 ml. EMEM containing 10% FCS and supplemented with 0, 20 or 60% day 3 thymus or spleen organ culture medium. After a 17 hour preculture at $37^{\circ}C$ each UC was centrifuged at 2,000 rpm for ten minutes and for those UC's designated "preculture to fresh" 6.3 ml. medium was withdrawn and replaced with 6.3 ml. fresh EMEM supplemented with 10% FCS. The cells were then resuspended by gentle agitation and 1 ml. aliquots made into vials containing either 4 μl. PHA in 100 μl saline or 100 μl saline. 100 μl of an erythrocyte preparation was then added to each vial and, after

gentle mixing, the vials were placed, loosely-capped, into the plastic box which was sealed, gassed and placed in the incubator. The cultures were terminated 74 hours after the addition of PHA and 24 hours after the addition of tritiated thymidine.

The results of this culture are shown in table 6.2. The spleen cells were found to be more responsive to PHA than the cells used in experiment (1). Renewing the medium after a 17 hour preculture was found to increase isotope incorporation in both PHA-stimulated and unstimulated cells. The increase in isotope uptake was greater in those PHA-stimulated cultures in which the cells had been precultured with either the thymus or spleen organ culture medium. The spleen organ medium had a greater potentiating effect in this respect than the thymus organ medium. With regard to those cells cultured without PHA, in which the medium was changed after 17 hours, very little difference was found in the amount of isotope incorporated by cells precultured with or without thymus and spleen organ culture medium. When either the thymus or the spleen organ culture medium was present throughout the duration of the culture isotope incorporation was progressively inhibited in both PHA-stimulated and unstimulated spleen cells as the concentration of organ medium supplement increased.

Table 6.2 Isotope incorporation in cultures of 2.8×10^6 thymectomized rat spleen cells following a 17 hour preculture with day 3 thymus or spleen OC16 medium, followed by either culture in fresh medium (preculture to fresh) or culture in unchanged medium (continuous culture).

Type of culture.	OC16 day 3 medium supplement (%)	Thymus medium			Spleen medium		
		4.0 µl PHA	0 µl PHA	SI	4.0 µl PHA	0 µl PHA	SI
		cpm., mean ± SD			cpm., mean ± SD		
"Preculture to fresh".	0	8,841 ± 1,244	2,282 ± 345	3.9	8,841 ± 1,244	2,282 ± 345	3.9
	20	9,189 ± 993	2,663 ± 511	3.5	12,185 ± 2,619	2,599 ± 204	4.7
	60	11,583 ± 616	2,479 ± 145	4.7	12,954 ± 2,319	2,028 ± 248	6.4
"Continuous"	0	4,588 ± 1,044	924 ± 55	5.0	4,588 ± 1,044	924 ± 55	5.0
	20	4,368 ± 597	597 ± 57	7.3	4,350 ± 638	596 ± 56	7.3
	60	2,694 ± 340	427 ± 11	6.3	1,631 ± 222	252 ± 3	6.5

Experiment (3) The effect of (A) a 17 hour preincubation with day 6 medium from thymus and spleen OC16, followed by culture in fresh medium, and (B) a 17 hour preincubation and continuous culture with day 6 medium from thymus and spleen OC16, on tritiated thymidine incorporation in PHA-stimulated and unstimulated thymectomized rat spleen cells.

This experiment was performed, in parallel, three days after experiment (2). 17.4×10^6 spleen cells from a 51 day old thymectomized rat were cultured exactly as described in experiment (2) except that the supplement was day 6 thymus and spleen OC16 medium.

The results of this experiment are shown in table 6.3. Replacing the medium after a 17 hour preculture was again found to result in an increased isotope uptake in both PHA-stimulated and unstimulated cells. This time, however, there was no potentiation of isotope incorporation in response to PHA in those cells precultured with organ culture medium. Little difference was found between the isotope incorporated by those cells precultured with various concentrations of organ medium. When day 6 organ medium was present throughout the culture, decreased tritiated thymidine incorporation was found.

Table 6.3 Isotope incorporation in cultures of 2.6×10^6 thymectomized rat spleen cells following a 17 hour preculture with day 6 thymus or spleen OC16 medium, followed by either culture in fresh medium (preculture to fresh) or culture in unchanged medium (continuous culture).

Type of culture	OC16 day 6 medium supplement (%)	Thymus medium			Spleen medium		
		4.0 µl PHA	0 µl PHA	SI	4.0 µl PHA	0 µl PHA	SI
		cpm., mean ± SD			cpm., mean ± SD		
"Preculture to fresh"	0	21,951 ± 1,159	3,220 ± 406	7	21,951 ± 1,159	3,220 ± 406	7
	20	17,489 ± 5,846	2,525 ± 510	7	20,380 ± 2,334	2,734 ± 337	7
	60	17,190 ± 3,403	2,348 ± 163	7	20,810 ± 3,981	2,200 ± 177	9
"Continuous"	0	12,590 ± 1,171	967 ± 133	13	12,590 ± 1,171	967 ± 133	13
	20	10,001 ± 685	954 ± 71	10	11,607 ± 2,944	1,065 ± 14	11
	60	4,549 ± 1,365	459 ± 38	10	10,554 ± 272	626 ± 24	17

Experiment (4) The effect of (A) a 17 hour preincubation with day 3 medium from thymus and spleen OC17, followed by culture in fresh medium, and (B) a 17 hour preincubation and continuous culture with day 3 medium from thymus and spleen OC17, on tritiated thymidine incorporation in PHA-stimulated and unstimulated rat thymus cells.

From the combined results of OC16, the transplantation of thymus explants after three and six days in organ culture and the lymphocyte cultures of experiments (2) and (3) it was considered that day 3 thymus organ culture medium was more likely to contain a thymic humoral factor than day 6 medium. Accordingly, day 3 medium was cultured with intact rat thymus and bone marrow cells, respectively, in experiments (4) and (5). The experimental design was the same as that of experiment (2).

18.0×10^6 thymus cells from a 49 day old rat were cultured in 6.6 ml of EMEM containing 10% FCS and supplemented with thymus or spleen day 3 OC17 medium.

The results of this experiment are shown in table 6.4. No difference in isotope incorporation was found between those cells cultured in unsupplemented medium whether the medium was renewed after 17 hours or left unchanged. Confirming the results of the previous lymphocyte cultures was the finding that both spleen and thymus organ medium inhibited isotope uptake in both PHA-stimulated and unstimulated cells in "continuous culture". The only cells showing an enhanced thymidine uptake were those cells precultured in 60% thymus organ medium. In this case both PHA-stimulated and unstimulated cells showed increased isotope incorporation.

Table 6.4 Isotope incorporation in cultures of 2.7×10^6 rat thymus cells following a 17 hour preculture with day 3 thymus or spleen OC17 medium, followed by either culture in fresh medium (preculture to fresh) or culture in unchanged medium (continuous culture).

Type of culture.	OC17 day 3 medium supplement. (%)	Thymus medium			Spleen medium		
		4.0 µl PHA	0 µl PHA	SI	4.0 µl PHA	0 µl PHA	SI
		cpm., mean ± SD			cpm., mean ± SD		
"Preculture to fresh"	0	5,183 ± 812	284 ± 56	18	5,183 ± 812	284 ± 56	18
	20	5,235 ± 940	312 ± 167	17	5,290 ± 800	263 ± 16	20
	60	7,617 ± 286	428 ± 212	18	4,837 ± 333	357 ± 78	14
"Continuous"	0	5,257 ± 1,528	316 ± 73	17	5,257 ± 1,528	316 ± 73	17
	20	3,952 ± 152	224 ± 58	18	3,196 ± 501	259 ± 113	12
	60	2,919 ± 672	254 ± 21	11	1,267 ± 132	161 ± 19	8

Experiment (5) The effect of (A) a 17 hour preincubation with day 3 medium from thymus and spleen OC18, followed by culture in fresh medium, and (B) a 17 hour preincubation and continuous culture with day 3 medium from thymus and spleen OC18, on tritiated thymidine incorporation in PHA-stimulated and unstimulated intact rat bone marrow cells.

Two different sera were used in organ culture 18. EMEM was supplemented with either 10% FCS or 10% ADRI rat serum. Thymus and spleen explants were cultured in both media and thymus and spleen organ medium containing either 10% FCS or 10% ADRI rat serum were compared in the lymphocyte culture.

16.9×10^6 intact rat bone marrow cells were cultured in plastic UC's containing 6.6 ml EMEM supplemented with either 10% FCS or ADRI rat serum and either thymus or spleen organ medium. After a 17 hour culture at 37°C the cells were centrifuged and in half the UC's containing FCS the cells were resuspended in fresh EMEM containing 10% FCS, whilst in the other half the cells were resuspended in unchanged medium. All the cells precultured in EMEM, containing 10% ADRI rat serum were resuspended in fresh EMEM containing 10% FCS. 1 ml aliquots of cells were then added to vials containing 4.0 µl. PHA in 100 µl. saline or 100 µl. saline, and 100 µl of an erythrocyte preparation. After culture with or without PHA for 50 hours, 1µCi of tritiated thymidine was added and the cells were harvested 23 hours later.

The results from this experiment are shown in tables 6.5 and 6.6. Replacing unsupplemented medium at 17 hours with fresh medium was found to have little or no effect on isotope incorporation in both PHA-stimulated and unstimulated cells. In continuous culture both PHA-stimulated and unstimulated cells showed increased isotope uptake in the presence of

organ medium. This increase appeared to be due to increased uptake in the unstimulated cells. A 17 hour preculture with medium supplements followed by culture in unsupplemented medium had little effect on the isotope uptake in PHA-stimulated and unstimulated bone marrow cells. The results from bone marrow cells precultured in EMEM supplemented with ADRI rat serum were similar to those of bone marrow cells precultured in medium containing FCS.

Table 6.5 Isotope incorporation in cultures of 2.6×10^6 intact rat bone marrow cells following a 17 hour preculture with day 3 thymus or spleen OC18 medium, containing 10% FCS, followed by either culture in fresh medium, containing 10% FCS (preculture to fresh) or culture in unchanged medium (continuous culture).

Type of culture.	OC18 day 3 medium supplement. (%)	Thymus medium			Spleen medium		
		4.0 µl PHA	0 µl PHA	SI	4.0 µl PHA	0 µl PHA	SI
		cpm., mean±SD			cpm., mean ± SD		
"Preculture to fresh"	0	3,015 ± 301	1,729 ± 179	1.7	3,015 ± 301	1,729 ± 179	1.7
	20	3,146 ± 255	1,615 ± 329	1.9	3,292 ± 159	1,823 ± 207	1.8
	60	4,121 ± 905	1.889 ± 92	2.2	3,529 ± 208	1,777 ± 291	2.0
"Continuous"	0	2,768 ± 245	1,528 ± 329	1.8	2,768 ± 245	1,528 ± 329	1.8
	20	3,318 ± 322	2,330 ± 234	1.4	4,908 ± 1,306	3,836 ± 675	1.3
	60	3,701 ± 77	2,726 ± 194	1.4	3,114 ± 209	2,564 ± 344	1.2

Table 6.6 Isotope incorporation in cultures of 2.6 x 10^6 intact rat bone marrow cells following a 17 hour preculture with day 3 thymus or spleen OC18 medium, containing 10% ADRI rat serum, followed by culture in fresh medium, containing 10% FCS.

Type of culture	OC18 day 3 medium supplement (%)	Thymus medium			Spleen medium		
		4.0 µl PHA	0 µl PHA	SI	4.0 µl PHA	0 µl PHA	SI
		cpm., mean ± SD			cpm., mean ± SD		
"Preculture to fresh"	0	2,362 ± 109	2,452 ± 386	1.0	2,362 ± 109	2,452 ± 386	1.0
	20	2,985 ± 114	2,699 ± 701	1.1	3,019 ± 201	2,265 ± 644	1.3
	60	3,119 ± 123	1,952 ± 383	1.6	3,594 ± 308	1,844 ± 574	1.9

CONCLUSIONS AND DISCUSSION

There was no indication from the results presented in this chapter that if a thymic factor was present in thymus organ culture media it was active in increasing the responsiveness of lymphoid cells to PHA.

When thymus cells and thymectomized rat spleen and bone marrow cells were cultured in the continuous presence of either thymus or spleen organ culture medium the isotope incorporated by PHA-stimulated and unstimulated cells was reduced. A similar result had been found in chapter five concerning the continuous presence of tissue extract in lymphocyte culture. The exception to this result was the finding that intact rat bone marrow cells incorporated more tritiated thymidine in the presence of thymus or spleen organ culture medium. Similar results were found with bone marrow cells in chapter five.

Greater isotope uptake occurred in PHA-stimulated and unstimulated spleen and bone marrow cell cultures, when, after a 17 hour preculture the cells were transferred to fresh medium. Similar results were found in chapters three and five. This increased isotope uptake was thought to be due to the removal of cell debris and other factors that inhibit tritiated thymidine incorporation in cultured lymphocytes.

When thymectomized rat spleen cells were precultured with day 3 thymus or spleen organ culture medium and then cultured in fresh medium (experiment 2), the isotope uptake in PHA-stimulated cells was greater than that of cells precultured without extract. As this potentiating activity was present in both thymus and spleen organ media maturation of lymphoid cells by a thymic-dependent mechanism could not be the explanation of the effect. The medium harvested at day 6 from OC16 was assayed in experiment (3) and found not to have this potentiating effect. The only other instance when isotope incorporation was increased by preculture with thymus organ media was in experiment (4) where thymus

cells were precultured with 60% day 3 OC17 medium. In this case, however, isotope uptake was increased in both PHA-stimulated and unstimulated cells.

Several reasons for the failure to obtain increased PHA responses by incubating lymphoid cells with thymic factors have been discussed in chapter five, and although relevant to the present chapter the reasons are not repeated here.

In summary, those cells considered to be responsible for thymic humoral factor production (epithelial cells) were thought to be viable during, at least, a three day organ culture as judged from histological and transplantation studies. Incubation of lymphoid cells with various concentrations of thymus organ culture medium, however, did not lead to increased responsiveness to PHA. No evidence was obtained to support a thymic-dependent maturation of lymphoid cells via a humoral factor.

GENERAL SUMMARY AND CONCLUSIONS

As outlined in chapter one and in the introduction to some of the experimental chapters 'thymic humoral factors' have been implicated in the roles of (a) increasing and maintaining the maturity of lymphoid cells, and (b) increasing the number of lymphoid cells. The object of this thesis was to study the role of thymic humoral factors in the maturation of rat lymphoid cells. From a survey of the available tests of thymic function the response, in vitro, of lymphoid cells to PHA was chosen because it offered several advantages over the other available assays. Firstly, individual rats could be tested several times during the course of an experiment and the result from each test would be independent of the other tests. This would not be the case with dermal delayed hypersensitivity reactions or allogeneic skin grafts in which cases the response would be modified by the prior exposure of the test animal to the particular antigen. Secondly, in addition to in vivo thymic reconstitution experiments it would be possible to investigate, in parallel, whether dissociated lymphoid cells could acquire, in vitro, responsiveness to PHA.

An extensive literature survey, summarized in chapter two, indicated that using 'standard culture techniques' the response of lymphoid cells to PHA could be considered to be a semi-quantitative measure of cell-mediated immune responsiveness. Nevertheless, as B cells have been shown to respond to PHA under certain conditions and in view of the uncommon use of erythrocytes in the lymphoid cell cultures it was considered necessary to examine, in some detail, whether T cells were responsive to PHA and B were not, in the whole blood and lymphocyte cultures used in this study. The thymic dependence of PHA transformation was confirmed using the whole blood technique and is described in

chapter four. The results show that, in the rat, neonatal thymectomy leads to a persistent lymphopenia and a drastically reduced responsiveness to PHA. Partial restoration of the PHA response and the lymphocyte count in thymectomized rats occurred following the transplantation of two syngeneic neonatal thymuses into each rat. No instances of 'spontaneous' restoration of PHA response was observed in any untreated thymectomized rat. Through the analysis of the PHA response and lymphocyte count in individual blood samples the whole blood PHA response was considered to be a good measure of the peripheral blood T cell population. The percentage of T cells in the blood of intact rats was calculated to be between 60 - 70%. In the experiments described in chapter three and subsequently in chapters five and six it was shown that neonatal thymectomy reduced the response of dissociated spleen cells to PHA. This, therefore, completed the base from which further investigations followed.

An appreciation of the effect of serum on lymphocyte transformation, or more specifically upon tritiated thymidine incorporation in transforming lymphocytes was necessary for several reasons. Firstly, because serum from different sources would be present in various concentrations in the lymphocyte cultures. Secondly, in experiments comparing whole blood lymphocyte transformation in intact, thymectomized and "treated" thymectomized rats it was important to know what effect, if any, the serum from these animals had on the amount of tritiated thymidine incorporated. Several constituents of normal serum (alpha-globulins etc) are known to inhibit tritiated thymidine incorporation and also it was possible that a thymic factor, present in the serum of intact rats, might increase the responsiveness of lymphoid cells to PHA. A study was therefore carried out to compare the effect of serum and plasma from intact and thymectomized rats on tritiated thymidine

incorporation in PHA-stimulated lymphocytes. These "serum studies", described in chapter three, found that there was no difference between the ability of serum from intact and thymectomized rats to depress or increase tritiated thymidine incorporation in rat blood or spleen cells responding to PHA.

The effect of the possible presence of a thymic factor in cell-free extracts of rat thymus and in medium obtained from thymus organ cultures was investigated and the results reported in chapters five and six respectively. In these experiments rat lymphoid cells were incubated for varying periods in the presence of the putative thymic factor before PHA was added to the culture. In some cultures the tissue extract or organ medium supplement was removed from the cells after a preculture, before PHA was added, and the culture continued in "extract-free" or "organ medium-free" medium. Cell free extracts of rat spleen and spleen organ culture medium were used to control the effect of the thymic factors. In the case of thymic extracts, increased PHA responsiveness was not consistently demonstrated following the incubation of various unfractionated lymphoid cell populations with thymic extracts. Several possible reasons why increased PHA responses were not obtained are discussed fully in chapter five. The most reproducible effect of both the thymic and splenic extracts was that of decreasing the amount of tritiated thymidine incorporated in PHA-stimulated and unstimulated cells cultured in the continuous presence of the extract. When the extract was removed from the cells after culture for various times and before the addition of PHA the isotope incorporated in response to PHA was either slightly increased or decreased compared to those cells precultured in the absence of extract. PHA-stimulated and unstimulated cells cultured with the higher concentrations of extract invariably showed a reduced isotope uptake. This reduction

was usually found to increase with increases in the concentration of extract. Similar results were obtained from the "organ culture studies". The cells thought to be responsible for the production of thymic humoral factors (epithelial cells) were shown, by histological and transplantation studies, to be viable at least to the end of a three day organ culture. Incubation of lymphoid cells with cell-free medium obtained from three-day and six-day thymus organ cultures, however, did not lead to increased responsiveness to PHA. Also, 40 injections of thymus organ culture medium given to each of 12 neonatally thymectomized rats over an eight week period restored neither the low blood lymphocyte count nor the poor responsiveness of whole blood cells to PHA.

Negative or equivocal results were therefore obtained from both the in vivo and in vitro experiments designed to investigate whether thymic humoral factors could increase the response of rat lymphoid cells to PHA. The experimental results described in this thesis, therefore, do not answer the question of whether thymic humoral factors are involved in the maturation of rat lymphoid cells. This research does not concur with several recent reports (discussed in chapter one) in which both mouse and human lymphoid cells have been shown to undergo maturation, assessed by several cirteria including mitogen responsiveness, under the influence of thymic humoral factors. However, there are studies in which increased PHA responsiveness was not demonstrated following incubation of unfractionated lymphoid cells with thymic factors. It therefore appears that, in the rat, responsiveness to PHA, whilst being a good marker for thymic-dependent cells, may not be induced in immature rat lymphoid cells or may not be induced in enough cells to be useful in assays of thymic factors using unfractionated lymphoid cells. Further studies in the rat to find a subpopulation of lymphoid cells which might become responsive to PHA under the influence of a thymic humoral factor would therefore be desirable.

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DECLARATION.

I declare that this thesis has been composed by myself and that the work was carried out by myself.

D. DREWITT